NOV
Medical Lib

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME XXXIV, NO. 5 NOVEMBER 1, 1921

PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1921, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917.
Authorized June 29, 1918.

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y.
H. D. DAKIN, Scarborough, N. Y.
LAFAYETTE B. MENDEL, New Haven, Conn.
DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of between 500 and 600 pages. The subscription price is $3.00 per volume to subscribers in the United States and Canada, payable in advance; $3.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y.
W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

CICATRIZATION OF WOUNDS.

XII. Factors Initiating Regeneration.

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 24, 1921.)

The nature of the factors which initiate cicatrization after an injury is not as yet exactly known. The resumption of cell proliferation in the wounded tissues of an adult animal may be attributed to the removal of resistance to growth, in consequence of the defect resulting from loss of tissue.[1] In other words, the removal of the products of growth, that is of a portion of the tissues, immediately reinaugurates the growth process, just as the removal of the products of a balanced chemical reaction at equilibrium immediately reinitiates the forward reaction.[2] This means that regeneration, being a direct consequence of the injury, is started by forces within the organism. But the same phenomenon may also be logically attributed to the action of an external factor. According to this hypothesis, the cells would be directly stimulated to growth and multiplication by forces without the organism, acting on tissues deprived of their natural protection by the injury.

I.

Effect of Protection against Irritation.

If regeneration is a direct consequence of the loss of tissue and initiated by an internal factor, the cicatrization of a wound protected against all external irritation must take place normally. But if this hypothesis be not true, the wound should not begin to cica-

[1] Welch, W. H., *Science*, 1897, v, 813.

[2] Robertson, T. B., Principles of biochemistry for students of medicine, agriculture and related sciences, Philadelphia, 1920, 482.

trize. It was observed in 1908 that the latent period of cicatrization of a wound dressed with dead connective tissue or plasma clot was abnormally prolonged. This fact suggested that regeneration was not initiated directly by the loss of tissue and that, if the surface of the wound were effectively protected against mechanical, chemical, and bacterial irritations, the setting in motion of the process of cicatrization would be indefinitely postponed. In order to ascertain in what measure the onset of regeneration could be delayed by adequate protection of the surface of the wound, five experiments were performed. Two circular wounds of equal size were made on the dorsal region of dogs, according to a technique previously described.[3,4] The control wound was covered with a paste containing chloramine-T in a concentration which had been shown to be non-irritating for the tissues, and to keep them in a sterile condition.[5] The experimental wound was dressed with subcutaneous connective tissue, excised from the lumbar region of a dog and kept in cold storage. Circular flaps, slightly larger than the wound and about 0.5 cm. thick, were prepared and fixed to the surface of the experimental wound by a few stitches. Both wounds were protected by a pad of dry gauze, sutured to the skin. Then the dressing was completed by a few other gauze pads, a large amount of cotton, a bandage, and a shirt. The animals were examined after a period of time varying from 13 to 25 days. As the examination involved the removal of the stitches holding the inner dressing to the skin, and also of the stitches fixed to the connective tissue placed on the wound, necessitating a considerable disturbance of the wound, the experiment was stopped after the second or third dressing.

The results of the five experiments are summarized in Table I. Experiment 1 was unsuccessful because the gauze pads slipped from the surface of the wounds and infection occurred. 14 days after the operation, no difference was found in the condition of both control and experimental wounds. Experiments 2 and 3 succeeded partly. The protection given to the wounds by the connective

[3] Carrel, Alexis, and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

[4] All operations were performed under ether anesthesia.

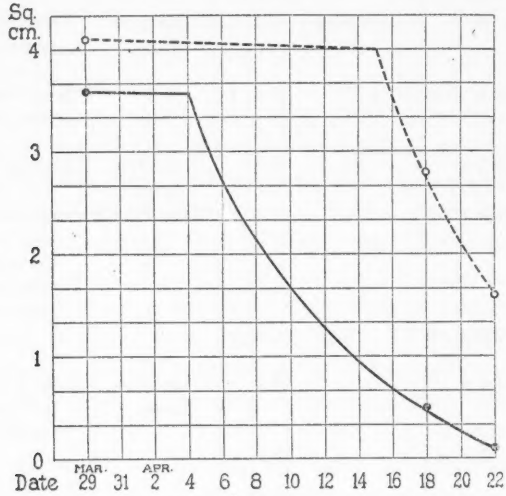
[5] Carrel, Alexis, du Noüy, P. L., and Carrel, Anne, *J. Exp. Med.*, 1917, xxvi, 279.

TABLE I.

Action of Connective Tissue Dressing on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
1	1	Apr. 5	4.0		Chloramine-T.	4.6		Connective tissue.	Connective tissue was heated at 56°C. for 3 hrs.
		" 19	1.9	16	"	1.4	1		Inner dressing slipped from both wounds.
2	2	Mar. 29	3.6		"	4.1		Connective tissue.	Text-fig. 1.
		Apr. 18	0.5	0	"	2.8	0	Chloramine-T.	Inner dressing slipped from both wounds. Connective tissue dressing partially disappeared.
		" 22	0.1	0	"	1.6	0	"	
3	3	Mar. 31	4.0		"	3.9		Connective tissue.	Inner dressing slipped. Infection of both wounds.
		Apr. 19	0.7	∞		2.8	∞		
4	4	Mar. 31	3.5		Chloramine-T.	4.7		Connective tissue.	Text-fig. 2. No displacement of dressing.
		Apr. 7	3.0	0	"				
		" 19	2.0	0	"	4.7	0	Dry gauze.	
		" 25	1.7	0	"	5.1		" "	Appearance of granulation tissue.
5	5	" 7	5.2		"	4.2		Connective tissue.	Text-fig. 3. Connective tissue heated at 56°C.
		" 20	3.3	0	"	4.0	0	Chloramine-T.	No displacement of dressing.
		" 25	3.0	0	"	4.5	0		Appearance of granulation tissue.

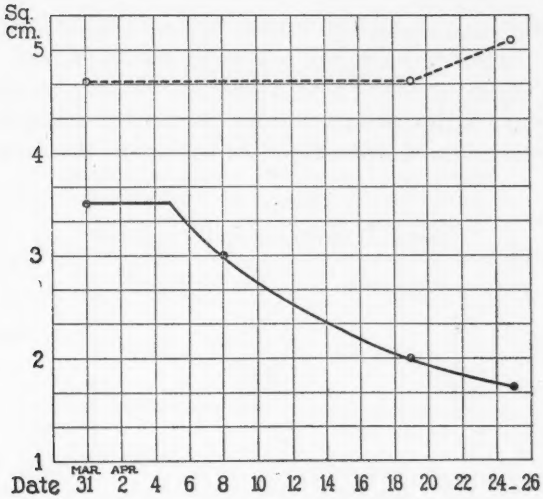
tissue dressing was incomplete. When the wounds were inspected for the first time, 19 and 20 days respectively after the operation, the experimental wound was no longer covered by the connective tissue dressing, and cicatrization had started. In Experiment 2, the curve expressing the progress of regeneration showed that the latent period had lasted very much longer in the wound protected by connective tissue than in the control wound (Text-fig. 1). The duration of the latent period was probably 17 days, while in the control it was 6.



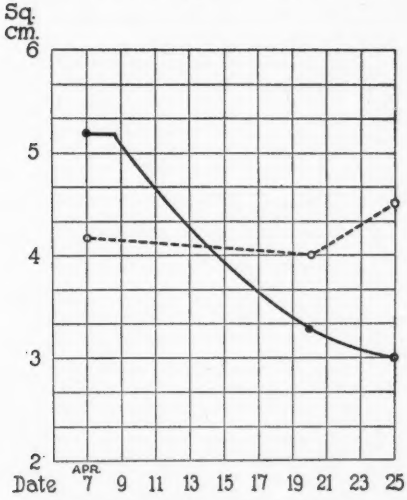
TEXT-FIG. 1. Experiment 2, Table I.

Although the connective tissue dressing did not remain at the surface of the wound for a long time, its effect was, however, manifest.

More significant results were obtained in Experiments 4 and 5. The connective tissue dressing remained exactly where it was applied and the surface of the wound was really protected against all outside irritation. The examination of the wounds was made 25 days after the operation in Experiment 4, and 18 days after the operation in Experiment 5. The period of contraction had not yet started and the area at that time was as large as at the time of the operation (Text-



TEXT-FIG. 2. Experiment 4, Table I.



TEXT-FIG. 3. Experiment 5, Table I.

figs. 2 and 3). It was a striking fact that a wound, effectively protected by a non-irritant dressing, did not show any evidence of cicatrization 25 days after the operation.

II.

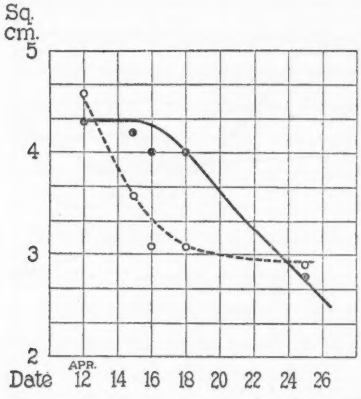
Effect of Irritants.

In a second series of experiments, it was investigated whether the application of mild irritants on the surface of the wound would

TABLE II.

Action of Turpentine on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
6	6	Apr. 12	4.3		Chloramine-T.	4.6		Turpentine.	Text-fig. 4.
		" 15	4.2	0	Dry gauze.	3.6	0	Dry gauze.	No displacement of dressing.
		" 16	4.0	0	" "	3.1	0	" "	
		" 18	4.0	0	" "	3.1	0	Turpentine.	
		" 25	2.8	0		2.9	1		



TEXT-FIG. 4. Experiment 6, Table II.

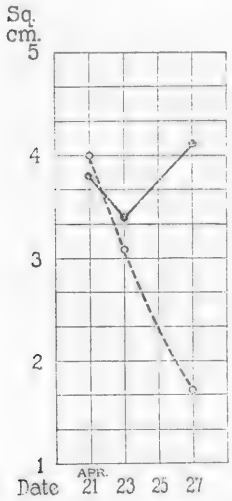
shorten the latent period. The experimental wound was covered by a gauze pad, soaked in turpentine and fixed to the edges of the skin by a few stitches, while the control wound was dressed with chloramine

paste. Both wounds were protected by gauze pads, sutured to the skin. The latent period of the experimental wound was very much shortened, lasting less than 2 days, while the latent period of

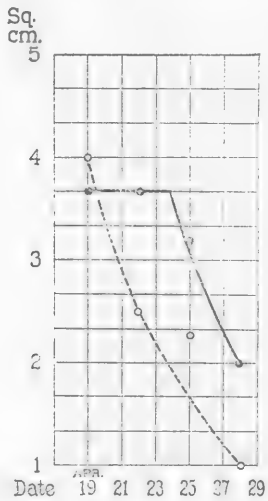
TABLE III.

Action of Chick Embryo Pulp on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
7	7	Apr. 21	3.8		Chloramine-T.	4.0		Chick embryo pulp.	Text-fig. 5.
		" 23	3.4	0	"	3.1	5	Dry gauze.	
		" 27	4.1	0	"	1.7	0	" "	



TEXT-FIG. 5. Experiment 7, Table III.



TEXT-FIG. 6. Experiment 10, Table IV.

the control wound lasted for about 5 or 6 days (Table II and Text-fig. 4).

In Experiment 7, chick embryo pulp was used instead of turpentine (Table III). 6 days after the operation, the contraction of the

control wound had not started. On the contrary, in the experimental wound contraction began after a very short time, less than 2 days

TABLE IV.

Action of Staphylococcic Infection on the Latent Period.

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	*sq. cm.*			*sq. cm.*			
8	8	Apr. 19	5.0		Chloramine-T.	4.0		1:100 dilution of staphylococcus suspension.	Mild infection; no edema; slight discharge.
		" 21		8	"		78	Dry gauze.	
		" 22	4.0	0	"	3.5	45	" "	
		" 25	3.2	0	"	3.3	24	" "	
		" 28	2.8	0	"	1.8	13	" "	
9	9	" 19	4.0		"	3.7		1:10 dilution of staphylococcus suspension.	
		" 21		4	"		∞	Dry gauze.	
		" 22	3.7	0	"	3.4	85	" "	
		" 25	3.4	0	"	3.9	1	" "	
		" 28	2.8	0	"	1.7	20	" "	
10	10	" 19	3.7		"	4.0		1:50 dilution of staphylococcus suspension.	Text-fig. 6.
		" 22	3.7	0	"	2.5	50	Dry gauze.	
		" 25	3.2	0	"	2.3	0	" "	
		" 28	2.0	0		1.0	41	" "	
11	11	" 21	4.0		Chloramine-T.	3.5		Pure staphylococcus cultures.	
		" 23	2.6	0	"	2.4	6	Dry gauze.	
		" 25	2.5	0	"	2.2	0	"	
		" 28	2.3	0	"	0			

(Text-fig. 5). 6 days after the operation, the area of the experimental wound was about 50 per cent smaller than that of the control wound.

In four experiments (Table IV), the wounds were infected with staphylococci. Varied dilutions of a 24 hour culture of staphylococci in bouillon were used for inoculation. The control wound was dressed with chloramine paste, while the experimental wound was inoculated with 0.05 cc. of the dilution of staphylococcic culture, and dressed with dry gauze. The wounds remained in a condition of slight infection without swelling of the edges or abundant suppuration. The duration of the latent period was decreased, and often reduced to less than 2 days, as shown in Experiment 10 (Text-fig. 6).

III.

SUMMARY.

As long as the wounds were protected by a connective tissue dressing against mechanical, chemical, and bacterial irritations, no evidence of cicatrization was found. The complete or partial failure of four experiments was due to the slipping of the inner dressing from the wound, mechanical irritation by the gauze, and infection. In the two experiments in which the connective tissue was maintained at the surface of the wound, there was no beginning of cicatrization, although 25 and 18 days respectively had elapsed since the operation, while in the control wound the duration of the latent period did not exceed 5 or 6 days. The experiments were interrupted after the second or third inspection, on account of the technical impossibility of again applying to the wounds a non-irritant dressing. It is probable that the wounds could have been kept for a much longer time in a condition of quiescence. While it is not known whether cicatrization could be prevented for an indefinite period, there is no doubt that the mechanism of regeneration is not set in motion at the usual time, when all external irritations are suppressed. It appears, therefore, that under ordinary conditions, cicatrization is not initiated by an internal factor.

On the contrary, the application of turpentine, chick embryo pulp, and staphylococci decreased markedly the length of the latent period, which was often reduced to less than 2 days. This fact demonstrated the importance of external factors in the initiation of cicatrization. It seems that the mechanism of regeneration has

become adapted to the ordinary conditions of life of the animals. A small wound will begin to cicatrize sooner if slightly infected, as practically always happens, than if it were thoroughly protected by a non-irritant dressing.

IV.

CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments, a wound, protected by a non-irritant dressing, shows no granulation tissue or beginning of contraction for 25 days at least.

2. Local application of certain irritants, such as turpentine, chick embryo pulp, and staphylococci, reduces the duration of the latent period to less than 2 days.

3. Regeneration is apparently initiated, not by an internal, but by an external factor.

REMOTE RESULTS OF COMPLETE HOMOTRANSPLANTATION OF THE CORNEA.

By ALBERT H. EBELING, M.D., and ANNE CARREL.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 34.

(Received for publication, June 6, 1921.)

Homoplastic transplantation of the cornea in its entire thickness has been attempted many times with practically constant failure. However, if this operation be performed under proper conditions, the transplanted fragments can remain transparent, and the curvatures of the cornea normal. This fact was demonstrated by Zirm, who resected part of a leucoma and substituted for it a flap of normal cornea taken from the enucleated eye of a boy.[1] About a year after the operation, the patient could see through the transplanted cornea. Although it had been implanted in scar tissue, the flap had remained transparent. Such a result is exceptional, and many other attempts have been unsuccessful. As the inadequacy of the technique was probably responsible for the failures of the operation, it would be important to improve the method of transplanting the cornea in such a way that it could be used as a routine procedure in cases of leucoma. The purpose of the experiments described in this paper was to develop a better technique for homoplastic transplantations.

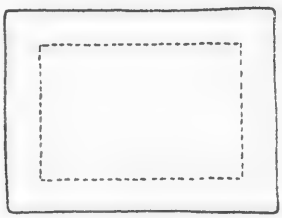
The technique consisted essentially in using a very large rectangular flap, fitted like the door of a safe into the edges of the corneal opening, and in fixing it securely in its position by stitches. This technique had been developed by Carrel in 1912, in the course of unpublished experiments on autotransplantation of the cornea. We attempted to adapt it to homoplastic transplantation, and to observe the results over a long period of time.

1. Preparation of the Eye.—The experiments were performed on cats. Cultures in bouillon were made of the secretion of the con-

[1] Zirm, E., *Arch. Ophth.*, 1906, lxiv, 580; *Wien. klin. Woch.*, 1907, xx, 61.

junctiva and only animals whose conjunctiva was found to be sterile were used. The hair covering the palpebra and the skin surrounding the eye were cut with scissors. Several days previous to the operation, the eye and the surrounding skin were washed with mercury bichloride at 1:5,000. Immediately before the operation, the surrounding skin and that of the palpebra were washed with alcohol, and afterwards tincture of iodine was applied.

2. Preparation of the Graft.—The graft was taken from the eye of a cat under ether anesthesia. The outline of a rectangular flap 6 by 8 mm. was traced on the surface of the cornea with a sharp cataract knife. The surface was incised to a depth corresponding to about half the cornea. Then the internal edge of the flap was dissected a distance of 1 mm. The anterior chamber was opened, the incision of the posterior part of the cornea continued with the scissors, the flap removed, and put in olive oil until it was used. Its anterior surface was 6 by 8 mm., while its posterior surface was only 4 by 6 mm. (Text-fig. 1).

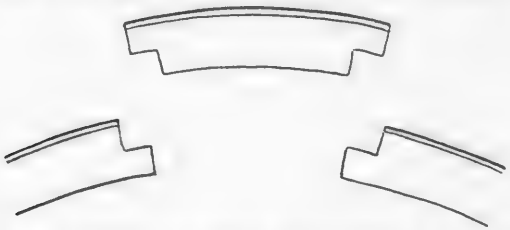


TEXT-FIG. 1. Anterior aspect of flap.

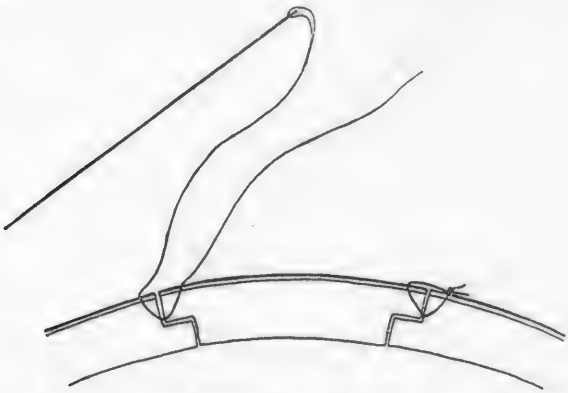
3. Resection of the Cornea and Transplantation.—The animal was etherized by the Meltzer-Auer method. The head was placed on a sand-bag and covered with a black silk towel, perforated in the center. The operating field was widely exposed by four small forceps fixed to the conjunctiva. The graft was placed on the surface of the cornea and the outline of the fragment to be resected was traced with the point of a cataract knife. Then the graft was replaced in olive oil. The incision of the cornea was made to a depth of about 1 mm. Then the internal edge was dissected for 1 mm., the anterior chamber was opened, and the fragment resected (Text-fig. 2). The graft was

immediately inserted in the opening, where it fitted the edges of the cornea exactly (Text-fig. 3).

4. Fixation of the Graft.—The suture of the graft to the cornea was made with straight needles, No. 16, and silk sterilized in vaseline. As the corneal tissue is very hard, prong-toothed dissecting forceps



TEXT-FIG. 2. Cross-section of corneal flap and opening in the cornea, showing step edges.

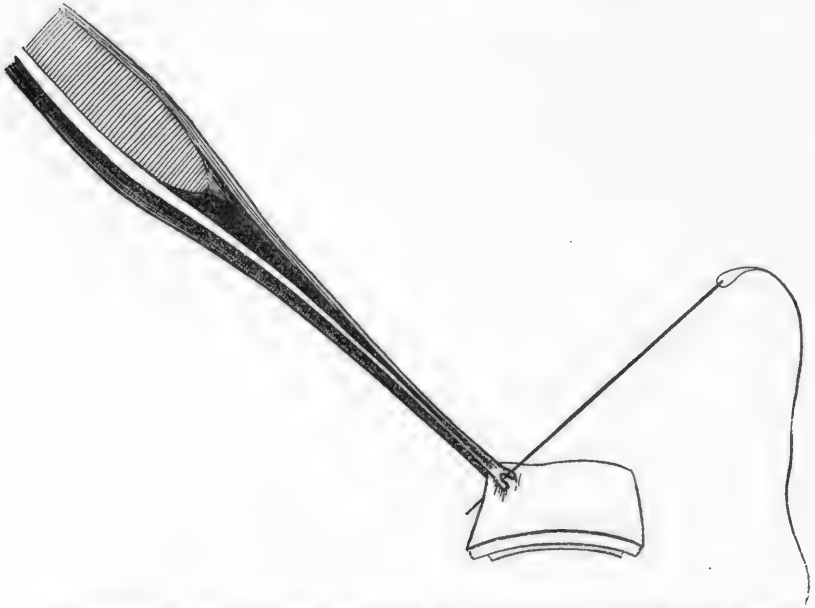


TEXT-FIG. 3. Transplanted flap in position, showing stitches passing through the superficial layers of the transplant and edge of opening in the cornea.

were used, the ends of which were bifurcated like a very small, double tined fork. The edge of the flap was seized by the forceps and the needle pushed through the tissue between the tines (Text-fig. 4). Only the superficial part of the cornea was caught by the needle. Each angle of the flap was fixed by one stitch. Another stitch was placed on the longer side of the rectangle. On account of the shape

of the edge, the incisions were closed tightly, and there was no leakage of fluid from the anterior chamber through the line of incision (Text-fig. 3).

5. Postoperative Treatment.—In previous experiments, it had been found that the dressing was often a cause of irritation. Therefore no dressing was applied. The animals kept the operated eye closed for several days after the transplantation. The corneal stitches fell out after a short time.



TEXT-FIG. 4. Prong-toothed dissecting forceps holding corneal flap and needle stuck through the tissue.

The experiments were made during an epidemic of distemper. Five cats were operated upon. Two died from distemper some time after the operations. Two others had a marked local infection of the eye and the cornea became completely opaque. In the fifth experiment (Cat 1, performed on May 20, 1919) the animal remained in good condition and the cornea transparent. A month later, the stitches had disappeared, the cicatrization was perfect, the cornea clear, and its curvatures did not appear to be modified. The iris was

not adherent to the cornea. There was a very small opaque spot near the inner canthus. Since that time, the animal has continued in the same condition. On May 26, 1921, the cornea was perfectly transparent, the only evidence of a previous operation being a hardly visible opaque spot on the internal part of the cornea (Fig. 1). An examination was made by Dr. W. B. Doherty, the results of which are as follows:

No line of demarcation to indicate the limitation of the corneal graft could be seen with a Zeiss loupe. Corneal epithelium prefectly even. No lack of corneal luster. Oblique illumination showed no inequalities of the corneal surface. Cornea was sensitive. Transplant perfectly transparent; no infiltration or vascularization. Ophthalmoscopic examination showed clear image of the fundus. Near the inner canthus and in the portion comprising the original cornea, there was a faint infiltration of the deeper layers with two or three small blood vessels. This opacity was of a striate appearance and gave the impression that it was produced by trauma during the operative procedure.

The result obtained in this experiment shows that the technique which was used allows a perfect reconstruction of the cornea, even when a very large fragment has been resected. The cutting of the flap is not difficult after a little training, and there is no doubt that the fixation of a flap fitted like the door of a safe to the step-edged cornea by suture increased the safety of the operation in a great measure. It is known that, in the previous attempts to transplant the entire thickness of the cornea, a disc of cornea was merely placed in an opening made with a trephine. In the operation performed by Zirm, the disc was maintained by two cross threads stitched to the conjunctiva. There is certainly a great advantage in cutting and fixing the flap in such a way that the anterior chamber is tightly closed, and that no displacement of the graft can take place.

SUMMARY.

1. A flap composed of the entire thickness of the cornea of a cat was transplanted to the cornea of another cat, and was found to be perfectly transparent 2 years after the operation.

2. The curvatures of the cornea appeared to be normal.

We wish to acknowledge our indebtedness to Dr. Doherty for the clinical examination of the eye and the report of the findings.

EXPLANATION OF PLATE 34.

FIG. 1. Cat 1. Right eye operated upon. Photograph taken May 31, 1921, 2 years and 11 days after the transplantation was made. The cornea appears normal. There is practically no difference between the cornea of the left eye and that of the right.

FIG. 1.

(Ebeling and Carrel: Complete homotransplantation of cornea.)

REMOTE RESULTS OF OPERATIONS ON THE PULMONARY ORIFICE OF THE HEART.

By ALEXIS CARREL, M. D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 6, 1921.)

In 1913, an attempt was made to develop a technique by which the size of the pulmonary orifice of the heart could be increased or decreased. The immediate results of the experiments showed that plastic operations on the wall of the pulmonary artery and its sigmoid valves caused little danger to the life of the animal, when a proper procedure was employed. The purpose of this paper is merely to examine the remote effects of the operations.

I.

EXPERIMENTAL.

In four experiments, the pulmonary orifice was enlarged by a vertical incision and suture of a flap of vessel.[1,2] In five others, the sigmoid valves were cauterized, incised, sutured, and the circumference of the orifice was shortened by stitching two of the valves together.[2,3,4]

1. Patching of the Pulmonary Orifice.—The operation consisted in suturing to the anterior side of the orifice a flap of a vein which permitted an increase in the circumference of the orifice after the wall had been incised.[1,2]

Experiment 1.—Black, female dog; medium sized, No. 1. October 23, 1913. The patch was a fragment of human aorta taken 24 hours previously from a cadaver and preserved in cold storage. The incision was made on the anterior side of the pulmonary artery. May, 1914. Animal still in normal condition. October 16. Died.

---

[1] Tuffier, T., and Carrel, A., *J. Exp. Med.*, 1914, xx, 3.

[2] All operations were performed under ether anesthesia.

[3] Carrel, A., *J. Exp. Med.*, 1914, xx, 9.

[4] Carrel, A., *Ann. Surg.*, 1914, lx, 1.

Autopsy.—Adhesions of the lungs to the thoracic wall. Heart surrounded by adhesions. Adhesions of the pericardium. Anterior side of the pulmonary artery covered by a layer of fibrous tissue. Surface of the intima of the pulmonary artery smooth, and sigmoid valves entirely normal. Above the anterior valve, depression of the wall about 8 mm. wide, 18 mm. long, and 2 mm. deep.

Experiment 2.—Black and white bulldog, medium sized, No. 2. October 31, 1913. The patch was made of a flap of dog jugular vein, preserved for 24 hours in cold storage. May 20, 1914. Animal normal. March 3, 1915. Died after an illness of several weeks.

Autopsy.—Adhesions of the lung to the thoracic wall. Fluid in the pleural cavity. Adhesions of the pericardium. A thick layer of fibrous tissue on the anterior part of the pulmonary artery and the upper part of the ventricle. Indistinct and narrow scar above the anterior sigmoid valve of the pulmonary artery. No apparent depression of the wall. Surface of the intima smooth. Sigmoid valves normal. Location of the patch detected by thickening of the wall.

Experiment 3.—White, male bulldog, No. 3. November 26, 1913. Patching of the pulmonary artery with a piece of dog vena cava, preserved for 1 month in cold storage. May, 1914. Animal normal. February 17, 1915. Died.

Autopsy.—No adhesions of the pericardium. No adhesions of the pulmonary artery to the aorta or the auricle. Connective tissue on the anterior part of the vessel thickened. No scar visible from the outside. Opening of the vessel along the posterior side. Sigmoid valves normal. No apparent insufficiency. Slight depression of the wall above the anterior valve. Surface smooth and scar very indistinct. Upper part of the scar 1.5 cm. above the sigmoid and hardly visible. Location of the patch detected only by a slight thickening of the wall.

Experiment 4.—White, female bulldog, No. 4. December 2, 1913. The incision of the pulmonary artery was patched by a flap of jugular vein, preserved in cold storage since November 28, 1913. May, 1914. Animal in good condition. December 21. Died.

Autopsy.—No adhesions of the pleura or the pericardium. However, a few loose adhesions at the posterior part of the heart. Anterior part of the pulmonary artery free from fibrous tissue. No scar on the external side. After opening of the artery, an indistinct and slightly depressed scar visible above the anterior sigmoid. Surface of the intima smooth. Patch located by a slight thickening of the wall.

2. Cauterization, Section, and Suture of the Sigmoid Valves.—After longitudinal opening of the artery, just above the orifice, the sigmoid valves were cauterized with the thermocautery, or they were severed and sutured, or two of them were stitched together.[2,3,4]

Experiment 5. Cauterization of the Sigmoid Valves.—Young brindle, female bulldog, No. 5. April 14, 1914. Cauterization of the edges of the left and right

sigmoid valves with the thermocautery. June, 1921. Dog in good condition. No murmur.

Experiment 6. Cauterization of the Sigmoid Valves.—White and yellow, male fox-terrier, No. 6. April 29, 1914. Cauterization of the anterior and the right valves. December 29, 1916. Died.

Autopsy.—Adhesions of the lungs to the pericardium. Heart enlarged. Adhesions of the pericardium and the pulmonary artery to the aorta and the left auricle. Fibrous tissue on anterior face of the pulmonary artery. Anterior sigmoid valve rigid and thickened, especially along its margin. Left posterior valve normal. Two holes about the size of a pin-head near the anterior insertion of the right valve. Scar of the incision of the arterial wall hardly visible, especially in the center.

Experiment 7. Cauterization of the Sigmoid Valves.—White fox-terrier, with brindle spots, No. 7. April 30, 1914. Cauterization of the posterior valves. Animal remained in good condition for 6 years and died of double pleuropneumonia, May 31, 1920.

Autopsy.—No adhesions of the lungs to the thoracic wall. Adhesions of the lungs to the pericardium. Pus in both pleural cavities. Loose adhesions of the pericardium at the anterior part of the heart. Linear scar at site of pulmonary incision. Sigmoid valves normal; no thickening of the edges. A few small holes in the wall of the posterior sigmoids near the margin.

Experiment 8. Section of a Sigmoid Valve.—Black, long haired dog, No. 8. March 10, 1914. Section of the right posterior valve with the scissors. Animal died during the period of the War. Heart, preserved in formaldehyde, examined May, 1920. Adhesions of the pericardium. Valve slightly rigid. No union of the incision; thickening of the edges.

Experiment 9. Stenosis of the Orifice by Stitching Both Posterior Valves.—Black and white, long haired, male dog, No. 9. March 12, 1914. The right and left sigmoid valves were united by a stitch at a distance of about 3 mm. from their insertion. Dog remained in good condition. May 10, 1918. Died of pneumonia.

Autopsy.—Pericardial adhesions. Margin of both posterior valves slightly thickened. Scar tissue on the wall of the artery at the insertion of the valves. No union of the valves and, therefore, no stenosis of the orifice.

Experiment 10. Section and Suture of the Right Sigmoid Valve.—Yellow and white, male fox-terrier, about 7 years old, No. 10. March 17, 1914. Right sigmoid valve was completely sectioned in the middle from the margin to the insertion. Approximation of the cut edges by a stitch. May 20. Slight diastolic murmur. June 8, 1920. Animal in good condition. No murmur. June, 1921. Same condition.

II.

DISCUSSION AND SUMMARY.

Eight animals died from 1 to 6 years after the operation, from undetermined diseases, or from pneumonia. Two animals are still alive 7 years after the operation.

1. Condition of the Pleural and Pericardial Cavities.—In the first experiments, extensive pleural and pericardial adhesions were observed 1 year and more after the patching of the pulmonary artery. There was also a great deal of fibrous tissue between the pulmonary artery, the aorta, and the left auricle. In the other experiments, the adhesions of the lungs, pleura, and pericardium were less marked. This was due to some improvements in the technique of handling the viscera. At the time of the operations, it was hoped that no pleural or pericardial adhesions would occur. Great care was taken not to injure the endothelial surfaces by rough handling or by sponging. No blood was allowed to flow into the pleural cavity. The surface of the pericardium was protected by fine silk membranes. The pleural cavity was occluded by thick pads made of cotton and Japanese silk. It seemed that the serous surfaces were almost completely protected against infection and mechanical irritation. The occurrence of primary pleurisy and pericarditis was prevented by this technique. But the development of adhesions in several of the experiments shows that the procedures for the handling of the viscera should be perfected.

2. Condition of the Arterial Wall.—In the experiments in which the orifice was patched, a slight dilatation of the artery was observed. It was not possible to ascertain from the specimen preserved in formaldehyde whether or not there was an insufficiency of the valves. It is probable that there was no leakage, as in none of these cases could any diastolic murmur be heard 6 months after the operation. The only animal which presented clinical evidence of pulmonary insufficiency died during the War. The normal condition of the pulmonary orifice was due to the incision which did not extend far enough on the ventricle, and to the power of redintegration possessed by an organ which is not diseased. The cicatrization of the grafted flap was excellent. Its outline could not be seen on the external side of the wall. Even after opening the artery, the transplant could not be

located easily. However, in Experiment 1 the anterior wall of the artery showed a depression about 7 or 8 mm. wide, 18 mm. long, and 2 or 3 mm. deep, behind and above the anterior valve. But the flap was made of human artery, and it is known that a heteroplastic graft always undergoes some dilatation. When transplants of dog tissue were used, no dilatation occurred and the location of the patch could hardly be detected. In Experiment 7, 6 years after the operation, the endothelial surface was smooth, glistening, and no scar could be seen. However, the upper and lower parts of the incision were marked by a slight depression of the wall. The presence of the patch was detected by a distinct thickening of the wall. Although the edges of the incision had not been sutured to the edges of the flap, the endothelial surface was quite smooth. A transverse section of the artery was made through the middle part of the flap in Experiment 4. It showed the width of the arterial opening and the way in which the transplant became adherent to the arterial wall. The examination of these four specimens demonstrated that, in spite of the unfavorable location of the graft, an excellent union had taken place. It showed that homoplastic or heteroplastic tissue can be transplanted onto the pulmonary artery as well as onto the smaller arteries. Where the arterial wall had simply been incised without interposition of a patch, a linear scar was always found. 6 years after the operation, the incision used in the course of an operation for cauterization of the sigmoid and sutured with heavy thread was transformed into a linear scar and the surface of the intima was quite smooth.

3. Condition of the Sigmoid Valves.—In three experiments, the sigmoid valves had been cauterized along their margin and their point of insertion in the artery. One of the animals was still living 7 years after the operation. There was no diastolic murmur. The other animals died 3 and 6 years after the operation. The valves were thin and transparent, and quite normal. However, one of the valves showed two holes, one near the base and the other near the margin.

The animal on which the section of the right posterior valve without suture was performed, died 2 or 3 years after the operation. The

edges of the incision had not united. They were thickened and the whole valve was rigid. The surface was rough and irregular.

No permanent result was obtained by the union of two sigmoid valves by a stitch. There was no stenosis of the orifice, and no union of the valves 4 years after the operation. The stitch had disappeared. There was some scar tissue at the common point of insertion of the posterior valves, which were more rigid and showed thickened edges. 7 years after the section and suture of a sigmoid had been performed, the animal was still living and in good health. No diastolic murmur could be detected.

GROWTH OF FIBROBLASTS AND HYDROGEN ION CONCENTRATION OF THE MEDIUM.

By ALBERT FISCHER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 6, 1921.)

The purpose of the experiments described in this paper was to determine the rôle played by the hydrogen ion concentration of the medium with regard to the growth of a strain of fibroblasts cultivated *in vitro* for a long period of time. The fibroblasts used were derived from fresh embryonic chick heart, from 1 or 2 months old strains of connective tissue, and from a strain cultivated for 9 years.[1] The cultures were prepared according to a technique previously described.[2] The method used for determining the hydrogen ion concentration has been described recently by Felton.[3]

I.

Method of Obtaining Variations in the Hydrogen Ion Concentration of the Media.

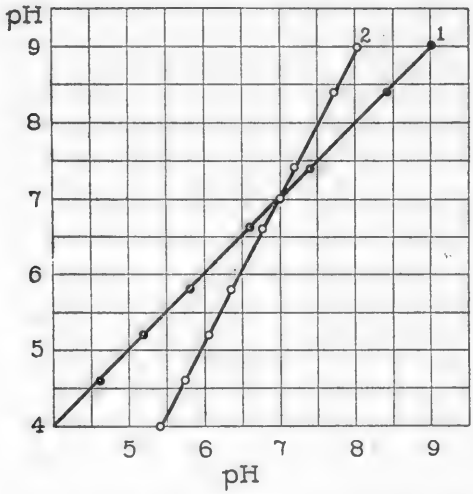
The variations in the hydrogen ion concentration of the media were obtained in the following ways.

1. Acid Solutions.—Solutions of acids were made in such concentrations that the addition of a small amount, usually 1 drop, to a certain amount of embryonic tissue juice or plasma would give the hydrogen ion concentration desired. A quantity of juice or plasma sufficient for a whole series of experiments was made in order to secure the same reaction in all. One of the disadvantages of this method was that plasma prepared in quantity underwent changes which resulted in decreasing or entirely preventing coagulation. A second disadvantage was that the embryonic tissue juice sometimes caused subsequent precipitation.

[1] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.
[2] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531–535.
[3] Felton, L. D., *J. Biol. Chem.*, 1921, xlvi, 299.

2. Phosphate Solutions.—Sörensen's standard phosphate solutions were added to embryonic tissue juice in equal parts, to obtain different hydrogen ion concentrations in the media. It was found that if equal parts of fresh embryonic tissue juice and sterile buffer solutions were mixed, for instance with a pH of 7, the growth obtained was nearly as extensive as that in the control in which the juice was diluted with Ringer solution. This showed that phosphate had no appreciable influence on the rate of growth.

A known hydrogen ion concentration was obtained by the following technique. Some preliminary experiments were made in order to find



TEXT-FIG. 1. The abscissæ indicate the pH obtained, and the ordinates the pH of the buffer solution added. Curve 1 represents the pH of Ringer solution, and Curve 2 the pH of embryonic tissue juice, to both of which are added the different standardized buffers as indicated by the ordinates.

out the pH of a mixture of embryonic juice and a given acid. Different numbers of drops of the respective solutions (acid or buffer) were added to tubes holding the same amounts of juice, and the hydrogen ion concentration was tested. These different known reactions of the juice obtained in this way gave a curve in which the amount of acid necessary to obtain a certain hydrogen ion concentration between the

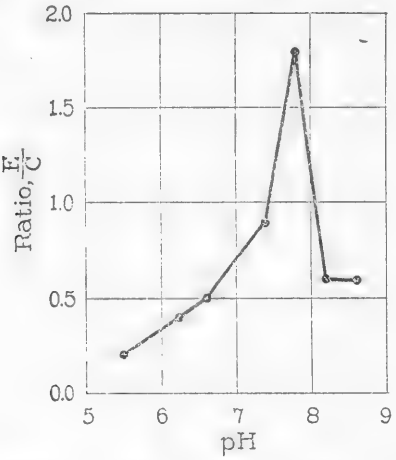
points found empirically could be calculated by interpolation (Text-fig. 1). Equal volumes of the standardized buffer solutions and of tissue juice were used. Upon ascertaining what pH resulted in the tissue juice when it was mixed with its own volume of buffer solution, the reactions obtained gave a straight line when a curve was plotted and it was possible to calculate readily which buffer should be used to produce the hydrogen ion concentration desired in the tissue juice (Text-fig. 1). Another test made in the same way was then necessary; namely, with the plasma-juice mixture. This had to be done rapidly in order to mix the indicator and juice-plasma before coagulation took place. In other words, when fibroblasts are to be cultivated in a medium of, let us say, pH 6, preliminary experiments for those particular media will show what buffer solutions should be added to the juice which will give pH 6, when combined with plasma. The test should be made in two steps for each experiment, one for the juice-buffer, and the other for the juice-buffer-plasma mixture, because of the slight differences in hydrogen ion concentration of the different juices and plasmas. The tissue juice may vary from pH 6.8 to 7.2, and the plasma from pH 7.4 to 8.

II.

Cultivation.

After the embryonic juice had been tested to determine its hydrogen ion concentration, it was mixed with equal parts of a buffer solution, calibrated pipettes being used to obtain the same sized drops. A selected culture was divided in two equal parts and washed in Ringer solution for about 30 seconds. One fragment was cultivated in a medium composed of 1 drop of plasma and 1 drop of a mixture of embryonic tissue juice and buffer solution. The other fragment, or the control, was cultivated in 1 drop of plasma and 1 drop of embryonic tissue juice to which had been added the same volume of Ringer solution instead of buffer solution. Mica cover-slips were used. 1 hour after the preparation of the culture, a drawing was made under the projectoscope. After 48 hours incubation, the outline of the new growth was drawn and calculations were made, by the method described by Ebeling.[2] When the purpose of the experiments was the study of the influence of the same hydrogen ion concentration for several genera-

tions, the culture was divided in two parts at each passage. One fragment was kept as control and the other placed in the experimental medium, care being taken to keep the other factors constant.



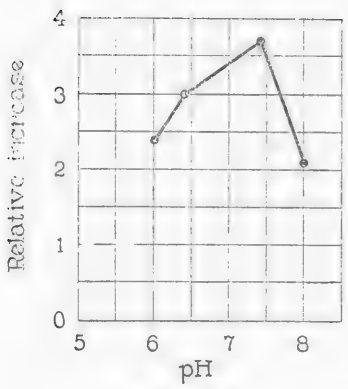
TEXT-FIG. 2. The different hydrogen ion concentrations of the media in which the fourth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth, $\frac{E}{C}$. The different hydrogen ion concentrations in the medium were obtained by adding phosphate buffer solutions to the extract.

III.

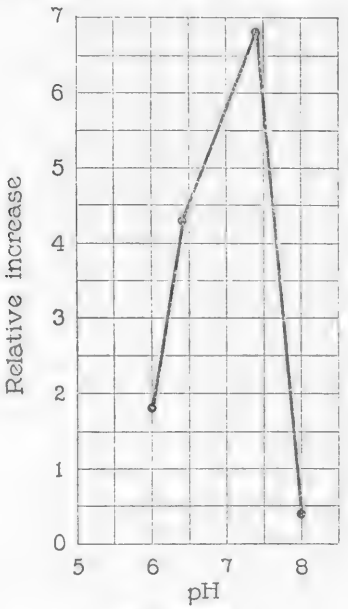
Rate of Growth.

When fibroblasts were cultivated in media containing different hydrogen ion concentrations varying from pH 5.5 to 8.5, a curve was obtained for the rate of growth with a distinct maximum between pH 7.4 and 7.8 (Text-figs. 2 to 4). The rate of growth decreased very rapidly with increasing hydrogen and hydroxyl ion concentrations, and the plotted curve was nearly symmetrical. The results obtained by using different acids, such as acetic, phosphoric, sulfuric, and hydrochloric, were much the same. It seemed that the anions did not have any remarkable influence on the growth. The curves reached a maximum at

pH 7.4 and fell very rapidly on both sides of this point, appearing always to be steeper on the alkaline side in spite of the fact that the resistance against the alkalinity was more marked. The same type of curve was found in all experiments. When the media were more alkaline or more acid, precipitation occurred, coagulation was interfered with, and it was impossible to draw any conclusions from the results. If the series of experiments were repeated by carrying the fibroblasts to a new medium containing the same hydrogen ion concentration as was used in the preliminary culture, it was observed that the absolute



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. The different hydrogen ion concentrations of the media in which the first passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

TEXT-FIG. 4. The different hydrogen ion concentrations of the media in which the fifth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

TABLE I.

Experiment No.	Culture No.	Buffer solution added.*	Juice-buffer mixture.	First passage.			Second passage.			Third passage.			Fourth passage.		
				Control.	Experiment.	Ratio, $\frac{E}{C}$.	Control.	Experiment.	Ratio, $\frac{E}{C}$.	Control.	Experiment.	Ratio, $\frac{E}{C}$.	Control.	Experiment.	Ratio, $\frac{E}{C}$.
		pH	*pH*												
1	264	4.0	5.5	7.0	0.4	0.6	6.2	3.1	0.5	3.0	2.7	0.9	6.1	1.7	0.2
2	265	4.6	6.0	5.4	0.1	0.1	6.3	2.4	0.3	6.6	3.0	0.4	6.5	0.9	0.1
3	266	5.2	6.2	3.1	7.3	0.4	16.5	4.7	0.2	3.0	1.7	0.5	6.6	3.0	0.4
4	267	5.8	6.6	1.8	1.5	0.7	8.7	4.1	0.4	4.6	3.3	0.7	3.0	1.7	0.5
5	268	6.6	7.2	4.7	3.9	0.8	12.4	1.8	0.1	4.2	5.1	1.2	5.8	3.4	0.5
6	269	7.0	7.4	10.8	10.8	1.0	12.2	11.9	0.9	4.8	6.8	1.4	7.3	6.6	0.9
7	270	7.4	7.8	10.4	6.6	0.6	4.3	5.1	1.1	9.9	12.1	1.2	2.1	3.9	1.8
8	271	8.4	8.2	11.8	4.5	0.3	12.1	7.9	0.6	10.3	8.1	0.7	3.7	2.3	0.6
9	272	9.0	8.6	7.2	6.3	0.8	11.1	7.7	0.7	12.7	8.4	0.6	7.6	4.9	0.6

* 1 cc. of buffer solution was added.

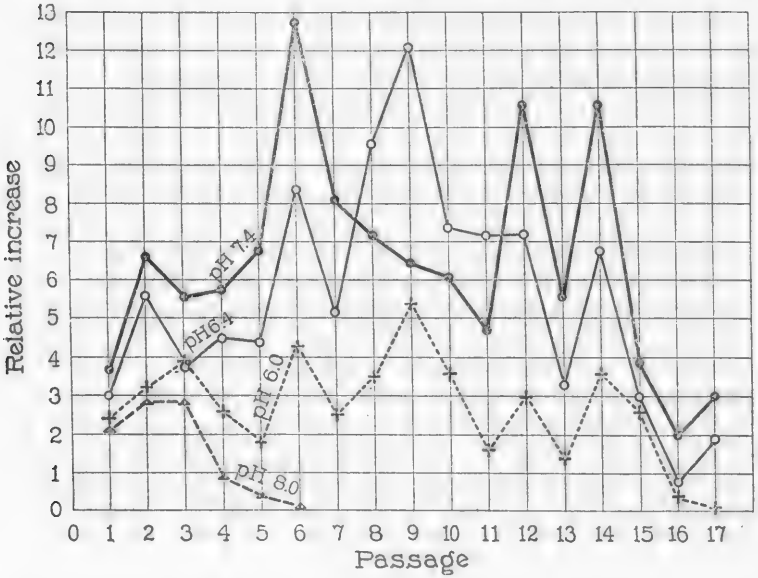
TABLE II.

Experiment No.	Culture No.	Buffer solution added.*	Juice-plasma mixture.	Relative increase. Passage No.																
				1	2	3	4		6	7	8	9	10	11	12	13	14	15	16	17
		pH	*pH*																	
1	375–1	4.0	6.0	2.4	3.2	3.9	2.6	1.8	4.3	2.5	3.5	5.4	3.6	1.6	3.0	1.4	3.6	2.6	0.4	0
2	375–2	5.8	6.4	3.0	5.6	3.7	4.5	4.3	8.3	5.2	9.6	12.1	7.3	7.2	7.2	3.3	6.8	3.0	0.8	1.9
3	375–3	7.0	7.4	3.7	6.6	5.6	5.8	6.8	12.7	8.1	7.2	6.5	6.1	4.7	10.6	5.6	10.6	3.9	2.0	3.0
4	375–4	9.0	8.0	2.1	2.8	2.8	0.9	0.4	0	0	0	0	0	0	0	0	0	0	0	0

* 1 cc. of buffer solution was added.

increase of new growth became less and less in the most alkaline and most acid media (see also Tables I and II and Text-fig. 5). The descent of the curve on both sides of the maximum became more abrupt in the succeeding generations because of the retardation of growth in the media with highest alkalinity and acidity. (Compare Text-fig. 3, which represents the first passage, and Text-fig. 4, which represents

the fifth passage.) By following the growth of fibroblasts, generation after generation, in media containing the same hydrogen ion concentration it was observed that in the highest acidity (pH 5.5) cell proliferation ceased after four to six passages. The fibroblasts showed more resistance to higher alkalinity, but at the highest (pH 8.5) they grew for about eight to ten passages. It may be seen from the curve (Text-fig. 2) that the growth of tissue at pH 5.5 was very near the reaction where



TEXT-FIG. 5. The growth of the four cultures of the experiments in Table II, expressed graphically.

no growth will take place, although at pH 8.5 it was further from this point, and growth was observed for more than ten passages. It may be assumed, therefore, that growth can continue for a long time in this hydrogen ion concentration, but with a smaller increase of cell proliferation. The optimum growth occurred between pH 7 and 7.8. This was the normal reaction of a mixture of plasma and embryonic tissue juice. It may be seen from the curves that a slight variation in the hydrogen ion concentration of the culture medium resulted in marked changes in

the rate of growth of fibroblasts. It is interesting to note that the differences were mostly of a quantitative nature. The only morphological change of the cells observed was that, in the acid media, they showed more vacuoles than in the alkaline.

IV.

CONCLUSIONS.

1. The rate of growth of fibroblasts is markedly modified by slight changes in the hydrogen ion concentration of the medium. The curves expressing the rate of growth in function of the hydrogen ion concentration of the medium are nearly symmetrical on both sides of the maximum.

2. The optimum growth of fibroblasts occurs at pH 7.4 to 7.8. A slight change from this reaction has a remarkable influence on the rate of growth.

3. Fibroblasts show more resistance to higher alkalinity than to higher acidity. They grew for only four to six generations in a medium having a pH of 5.5, and for more than ten generations in one of 8.5.

4. The influence of different hydrogen ion concentrations on fibroblasts was only of a quantitative nature.

I wish to acknowledge my appreciation to Dr. Felton for his advice regarding his own method for measuring the hydrogen ion concentration of small amounts of fluid.

STUDIES ON BACTERIAL NUTRITION.

III. Plant Tissue, as a Source of Growth Accessory Substances, in the Cultivation of Bacillus influenzæ.

By THEODOR THJÖTTA, M.D., and O. T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 1, 1921.)

The preceding papers (1–3) in this series have dealt with the nature and action of the substances contained in blood which are of importance in the growth of *Bacillus influenzæ*.

Search for substances other than the hemoglobin of warm blooded animals, which might be capable of stimulating growth of *B. influenzæ*, has been made by several investigators. Davis (4) found that substances similar in their function to hemoglobin, namely hemocyanin, hemoerythrin echinochrom, did not sustain growth. He was unable to find any growth-inducing action in substances which readily give up oxygen, such as hydrogen peroxide and colloidal platinum, and among the various salts and organic preparations of iron he found none capable of replacing hemoglobin. Davis (5) has confirmed the observation of Ghon and von Preyss (6) that *B. influenzæ* will grow on hematin agar only in the presence of dead or living bacteria. Davis points out that while hemoglobin in plain agar yields growth, still more abundant multiplication occurs when hemoglobin is associated with bacteria or tissues, either plant or animal, and especially if these are living. However, he states that plant and animal tissues, and bacteria by themselves will not sustain growth.

Olsen (7), studying the effect of blood on growth of Pfeiffer's bacillus, tested the growth-inducing action of various constituents of blood and derivatives of hemoglobin. He found that serum and ether extracts of washed blood corpuscles were ineffective. He observed, further, that hemoglobin and methemoglobin were capable of supporting growth, while hematin and hemin, on the other hand, were effective only in association with other bacteria. This author suggests that hemoglobin acts as a catalytic agent in rendering oxygen available for the bacilli.

In a recent publication on the nature of the effect of blood pigment upon the growth of *B. influenzæ*, Fildes (8) favors the view that the iron-containing pigments function as catalysts in accelerating the transfer of oxygen to the bacilli. In interpreting the relative feebleness of growth on unchanged blood compared

to that on changed blood, Fildes attributes the growth differences to the fact that the oxygen affinity of unchanged hemoglobin may, by its own avidity, divert the oxygen from the bacilli, while physiologically inactive derivatives cannot intercept the oxygen liberated by the catalytic action of the iron in these pigments. In his experiments, however, Fildes never observed the occurrence of growth of *B. influenzæ* in the total absence of blood pigment.

In preceding papers (1–3) the authors have pointed out that the hemophilic bacilli, of which Pfeiffer's bacillus serves as a type, require for their growth two distinct and separable substances, both of which are present in blood and neither of which alone suffices. These substances are (*a*) a vitamine-like substance which can be extracted from red blood corpuscles, and from yeast and vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (*b*) a so called X substance which is also present in red blood cells, is heat-stable and acts in minute amounts.

The present paper concerns itself with the nature of this X substance in blood. It will be shown that this X factor can be derived also from sources other than blood, just as in a previous paper it was shown that the V factor can be supplied apart from animal tissue. That both the V and X factors exist in combination in nature will be shown by the fact that *Bacillus influenzæ* will grow in blood-free medium provided certain plant tissues are added.

EXPERIMENTAL.

Presence of the X Substance in Blood and Blood Derivatives.

In a previous paper (3) it has been pointed out that the X factor, which constitutes one of the growth essentials of *Bacillus influenzæ*, occurs in highest concentration in the cellular fraction of blood, and that the minimal effective amount is extremely small. In fact, the quantity of the X factor carried over in a loopful of the supernatant fluid of a blood broth culture of *Bacillus influenzæ* is sufficient to induce growth in yeast extract broth; that is, in the presence of the supplementing vitamine-like substance, the V factor.

Concentration of Accessory Substances in Blood Essential for Growth of Bacillus influenzæ.—From Table I, in which is shown the minimum concentration of hemoglobin permitting growth of *Bacillus influenzæ* in broth with and without yeast extract, the relationship of the two factors, V and X, is again evident. While the source of the blood

in each instance is different, the two preparations of hemoglobin illustrate in a comparative way the minimal effective ratio between these two factors in the same preparation, and the extraordinarily minute quantity of the X substance which suffices for growth in the presence of an excess of the vitamine-like factor from yeast. The hemoglobin derived from laked blood cells was physiologically active; the crystalline hemoglobin had lost its oxygen-carrying capacity, although it still retained the characteristic absorption bands on spectroscopic examination. The latter preparation was devoid of

TABLE I.

Concentrations of Hemoglobin Essential for Growth of B. influenzæ.

Concentration of hemoglobin in plain broth.*	Hemoglobin† from laked red blood cells.		Crystalline hemoglobin.‡	
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
1 : 10	++	++	—	++
1 : 100	++	++	—	++
1 : 1,000	++	++	—	++
1 : 10,000	+	++	—	++
1 : 100,000	—	++	—	++
1 : 1,000,000	—	+	—	++
1 : 2,000,000			—	+

* All tubes containing 5 cc. total volume were inoculated with 0.05 cc. of yeast extract broth culture of *B. influenzæ*. Plain broth without hemoglobin or yeast extract, and yeast extract broth alone served as controls and showed no growth under the same conditions of seeding.

† Hemoglobin from rabbit blood determined gasometrically by Van Slyke's method (Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127).

‡ Crystals of hemoglobin prepared from ox blood.

++ indicates marked growth; + moderate growth; — no growth.

the more labile vitamine-like substance as shown by the fact that of itself it was incapable of supporting growth even in the highest concentration. When complemented by the V factor in yeast extract, however, a 1:2,000,000 dilution of crystalline hemoglobin sufficed to stimulate growth of *Bacillus influenzæ*. On the other hand, in the medium containing the solution of freshly laked blood cells both factors were present in amounts sufficient for growth up to a certain dilution, beyond which the native V factor was exhausted and the

presence of the residual X substance could be demonstrated only by adding the vitamine principle from yeast. Under the latter circumstances the X factor was still effective in concentrations of hemoglobin as small as 1:1,000,000.

Davis found that hemoglobin from human blood was active in promoting growth of *Bacillus influenzæ* in dilutions as high as 1:180,000. Fildes (8) could not confirm the growth activity of sheep blood in these high dilutions. The sources of error suggested by Fildes, namely the carrying over of blood pigment from tube to tube, or with the inoculum from the culture itself, have been eliminated in the present study by the use of a separate pipette for each dilution, and by seeding all tubes with a small inoculum from a yeast extract broth culture known to contain only the minimal effective dose of the X substance. Furthermore, in the present experiments, broth rather than agar was used as the medium of choice. It seems not impossible, therefore, that these differences in methods, together with recognition of the need of the separation and titration of the two component factors individually, may account for the variations in question. Individual specimens of blood from the same or different species and corresponding derivatives of hemoglobin will of necessity vary in one or the other of these factors, particularly in their content of the V factor, so that comparisons of this sort express only relative values. Of more importance is the quantitative relationship, and the interdependence of these two factors in a given specimen, and the need, in testing for either substance in blood derivatives, of recognizing that the more labile vitamine-like factor may be destroyed in the preparation of the material tested, or lost by dilution without a corresponding destruction or loss of the X substance.

Heat Stability of the X Substance in Blood.—Sufficient data have been presented in the preceding papers of this series to establish the thermostability of the X substance. Unlike the V factor, the X substance in blood, blood extracts, and crystalline hemoglobin resists autoclaving at 120°C. for 45 minutes. Further evidence of the resistance of the X substance to heat is found in the fact that blood charcoal, which reacts positively to the benzidine test, retains the ability to function as the X factor in 10 per cent yeast extract broth; that is, to support growth of Pfeiffer's bacillus in the presence of the supplementing V factor.

Benzidine Reaction.—Olsen (7) states that both the guaiac and benzidine reactions go hand in hand with the ability of blood derivatives to support growth of *Bacillus influenzæ*. This color reaction for the demonstration of the so called peroxidases has been found to parallel the presence of the X substance in blood. The question as to whether the positive benzidine-reacting substances and the X substance in blood are one and the same will be discussed later in describing the occurrence of these substances in plant tissue. It need only be remarked here, that in blood at least, the benzidine-reacting substances exhibit a marked resistance to heat, and in this instance again parallel the behavior of the X substance.

TABLE II.

Absorption by Bone Charcoal of the X Substance in Crystalline Hemoglobin.

10 per cent yeast extract broth 5 cc.* plus	Before absorption.		After absorption.	
	Benzidine test.	Growth-inducing action.	Benzidine test.	Growth-inducing action.
Crystalline hemoglobin, 1 : 1,000.....	++	++	—	—
Bone charcoal.....	—	—	++	++

* Inoculated with 0.05 cc. of 18 hour yeast extract broth culture of *B. influenzæ*.
++ indicates positive benzidine test or marked growth.

Absorption of the X Substance.—It has been noted in the preceding paper of this series that the V factor in yeast extract can be completely removed from solution by absorption with bone charcoal. Similarly it has been found that the X substance can be absorbed from solutions of crystalline hemoglobin by this agent. The solution of hemoglobin after absorption no longer reacts positively to the benzidine test, and has suffered loss of the X substance as evidenced by the lack of any growth-inducing property.

From Table II it is evident that bone charcoal can absorb the X substance from solutions of crystalline hemoglobin. Absorption is facilitated by heat, and is related to the concentration of the X substance in solution and to the time allowed for the reaction. It is of interest to observe that the original bone charcoal failed to give the benzidine test, but, after absorption, reacted positively and had

itself acquired the X substance, as shown by its growth-promoting action in 10 per cent yeast extract broth. The supernatant solution of hemoglobin after absorption had lost both its benzidine-reacting and growth-promoting properties as shown in Table II.

In view of these facts, an attempt was made to determine whether the X substance present in blood might also be found in other proteins of animal origin, such as crystalline egg albumin, or in protein split products such as peptone, erepton, and "aminoids." None of these substances, however, gave a positive benzidine test and none could be substituted for the X factor in supplying the cultural needs of this organism. In these experiments the V factor was always supplied by the addition of yeast extract to the medium, so that if the X factor were present in any test substance, the growth requirements would be complete. Furthermore, lipoidal extracts of heart muscle with and without cholesterol, as described by Noguchi, were tried under similar conditions without result.

Occurrence of the Growth Accessory Substances, X and V, in Plant Tissue.

In the course of these experiments it was observed, as already noted, that a striking parallelism seemed to exist between the presence of the benzidine reaction in blood derivatives and the ability of these same substances to promote growth of *Bacillus influenzæ*. This fact is not final evidence that the reacting substances are necessarily the same in both instances, but suggests rather that this color reaction for the so called peroxidases may serve as an indicator of the presence of the X factor in tissues other than those of animal origin. In the search for the X substance in vegetables, the potato was selected first, since it is known to contain peroxidases and catalysts and also both the fat-soluble A and the water-soluble B vitamines. For these reasons it was thought possible that raw potato would furnish both the V factor and the X substance. This was found to be the case by the luxuriant and continued growth of the bacilli which occurs in blood-free medium containing pieces of sterile raw potato.

Technique.—While no special attempt has been made to devise a precise method for obtaining pieces of sterile potato, and while

modifications will naturally suggest themselves to those trained in bacteriological technique, the following procedure is the one originally adopted. It is desirable to select a potato without surface abrasions or imperfections, and for this reason an old potato is preferable because of the thicker skin protection. Possibly differences in the content of the vitamine and the X substances in new and old potatoes exist, but these have not been determined. After thorough cleansing of the outer surface in running water, the skin is dried and well charred with a red-hot searing iron in a broad band encircling the potato. Through this seared band the potato is cut or broken open and from the inner portions small pieces are removed with a sterile scalpel and placed in sterile petri dishes, care being taken to avoid touching the margins or piercing the outer surface of the potato. The sections removed in this fashion are divided into pieces of suitable size and dropped into tubes of plain broth.

Oxidizing Enzymes of Potato.—It is well known that in plant, as well as in animal tissues, substances concerned with physiological oxidation and reduction are widely distributed. Scrapings from fresh potato exposed to the air rapidly change color as a result of oxidation processes. The benzidine reagent in the presence of hydrogen peroxide gives a blue color when applied to the cut surface of potato, the so called peroxidase reaction. Hydrogen peroxide alone applied in a similar way is rapidly decomposed with the liberation of gaseous oxygen through the action of a catalase in the potato. In studying the oxidase, peroxidase, and catalase of potato and other vegetables, Falk, McGuire, and Blount (9) noted that the enzyme reactions were destroyed by heating to boiling for several minutes. They observed that there was no well defined hydrogen ion concentration for maximum action of the vegetable enzymes, but that on the average the optimal zone lay between pH 7 and 10. According to the work of Cohn, Gross, and Johnson (10) on the isoelectric points of the proteins in certain vegetable juices, the natural hydrogen ion concentration of potato is pH 6 to 7.

Sterile raw potato possesses the property of slowly reducing methylene blue in solutions of phosphates ($\frac{M}{15}$, pH 7.5) from which atmospheric oxygen has been excluded by a vaseline seal. It has been noted

that, in hemoglobin, blood, or yeast extract broth under aerobic conditions, growth appears first in the upper layers of the medium where the oxygen tension is greatest. On the other hand, in broth containing raw potato under seal, growth of *Bacillus influenzæ* occurs early in the depths of the medium about the vegetable. Although the natural reaction of potato is acid (pH 6 to 7), and despite the presence of active enzymes, the use of raw potato in well buffered broth (pH 7.8) does not necessitate further adjustment of the reaction for growth of *Bacillus influenzæ*. In the cultivation of organisms more sensitive to reaction changes, the acidity developing in potato medium requires readjustment.

TABLE III.

Effect of Heat on the Growth Accessory Substances of Potato.

Inoculum, 0.05 cc. of yeast extract broth culture of *B. influenzae*.	Potato in plain broth.				Controls; plain broth.	
	Unheated.		Autoclaved at 120°C. for 45 min.			
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
Type* A.....	++	++	—	++	—	—
" B.....	++	++	—	++	—	—
" C.....	++	++	—	++	—	—
" D.....	++	++	—	++	—	—

* Types A, B, C, and D refer to the biological classification of Stillman and Bourn (Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665).

++ indicates marked growth; — no growth.

The nature and interaction of these vegetable enzymes are too complex and too little understood to warrant any interpretation of their possible significance in the growth of *Bacillus influenzæ* in media containing raw potato. The assumption that they may function as catalysts in facilitating the transfer of oxygen to the bacilli is perhaps justified by the fact that in potato broth, under vaseline seal, growth occurs in the depths of the culture about the vegetable tissue.

Heat Stability of the Accessory Substances in Potato.—From Table III it is evident that potato contains both the X and V factors requisite for growth of *Bacillus influenzæ*, since small pieces of the fresh sterile

vegetable in plain broth suffice to stimulate rapid multiplication. Under these conditions, growth is not conditioned by the carrying over of either factor with the inoculum. Repeated experiments have amply confirmed these observations, and their validity is further established by the fact that growth in unheated potato broth is not limited merely to the first transfer, as is the case in yeast extract broth, but may be continued by loop inoculation from tube to tube.

In studying the effect of heat on the growth accessory substances of potato the interesting fact is brought out that the two factors, X and V, react to temperature in the same manner as the similar substances in blood. The vitamine-like principle is less resistant to heat than the X substance (Table III). Potato broth exposed in the autoclave to a temperature of 120°C. for 45 minutes is no longer capable of supporting growth of *Bacillus influenzæ*. That the V factor is destroyed in the heating and that the more resistant X substance is left unimpaired is shown by the fact that the autoclaved medium can be reactivated by the addition of fresh active yeast extract.

Further evidence that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood for its peculiar nutritive requirements is afforded by the fact that *Bacillus influenzæ* will grow in the complete absence of blood derivatives, meat extractives, and animal peptones. The addition of sterile raw potato to Uschinsky's synthetic medium containing asparagine and ammonium lactate suffices to support growth of *Bacillus influenzæ*. A simpler medium of unheated potato in plain buffer solutions of sodium and potassium phosphate ($\frac{M}{15}$, pH 7.5) fulfills the necessary growth requirements of this organism. It is evident then that potato contains both the V and X substances and that these factors together with the native protein and carbohydrate of potato can replace in media blood pigment and tissue derivatives from animal sources.

In the absence of knowledge of the chemical nature of the X substance and in view of the chemical complexity of the tissues in which it occurs, the identity of the X factor must remain a matter of more or less conjecture. In blood, this substance seems to be associated with the iron-containing pigment, but attempts to substitute inorganic, organic, and colloidal forms of iron have been unsuccessful. The

occurrence of the X factor in potato, as well as in blood, suggests that this substance, or substances reacting similarly, may be commonly present in plant and animal tissue. Other vegetables have not been tested; banana, however, has been used and found to contain the essential growth factors. It is interesting to note that although furnishing the necessary growth accessory substances, banana fails to react positively to the benzidine tests. It possesses, however, a markedly active catalase, as evidenced by the evolution of gas when hydrogen peroxide is applied to the cut surface.

DISCUSSION.

The importance in animal nutrition of the presence in foodstuffs of growth accessory substances is now fully appreciated. There is a growing realization among biologists that this peculiar sensitiveness to the want of some particular substance in small amounts is not limited merely to the higher animals. In microbiology this principle finds its earliest expression in the work of Wildiers (11) (1901) on "bios," a substance extractable from yeast cells which exerts an accelerating influence upon the growth of yeast. Bottomley (12) has extracted from decomposing peat, substances which stimulate plant growth, and to which he has given the name "auximones."

The fact is also gaining recognition that bacteria require for growth not merely carbohydrates, proteins, and their derivatives suitably combined in a medium of optimal reaction. Many of the more difficultly cultivable microorganisms are sensitive to the lack in ordinary media of growth accessory substances allied perhaps to those of animal diet. The application of this principle to culture media has given rise to various modifications in the methods of preparation, each of which seeks to preserve or add certain substances, the presence or absence of which is recognized only by the growth-promoting value of the medium for a particular species. No group of organisms exhibits a more striking sensitiveness in this regard than the so called hemophilic variety.

The observations of Thjötta (2) on the multiplication of Pfeiffer's bacillus in hemoglobin-free medium have demonstrated that substances of bacterial origin can replace the growth-inducing factors of blood. It has been pointed out (1, 3) that there are two distinct and separable

substances in blood essential to the growth of this bacillus—a vitamine-like principle, the so called V factor, and a second substance, the so called X factor. Both of these substances are found in highest concentration in the cellular fraction of blood. They are both requisite for growth of hemophilic bacilli, and each is separately inactive and without effect. They differ in character one from the other in ways suggestive of the nature of their separate functions. The vitamine-like substance, the so called V factor, can be extracted from red blood corpuscles, from yeast and vegetable cells; it is relatively heat-labile, readily absorbed from solution by charcoal, required in greater concentration than the X factor, and resembles the known vitamines in its growth-promoting action. The second substance, the so called X factor in blood, is heat-stable, present in greatest concentration in red blood cells, absorbed from solution by charcoal, and effective in such minute amounts as to suggest a catalytic nature.

This dual function of blood in fulfilling the growth requirements of *Bacillus influenzæ* has been appreciated though less fully analyzed by previous observers. Davis (5), particularly, has directed attention to the fact that for maximum development of hemophilic bacteria both pure hemoglobin and a second vitamine-like substance are essential. He suggests that these two principles in blood may be analogous to the fat-soluble A and water-soluble B vitamines. In the separation of blood pigment from the other constituents of peptic digests of blood, Fildes (8) observed that neither the clear, colorless filtrate nor the precipitated pigment alone could support growth of *Bacillus influenzæ*. Combination of these two factors in media, however, afforded conditions favorable to growth.

Bacillus influenzæ has heretofore been considered an obligate hemophile. The present studies indicate, however, that the hemophilic property of this group of organisms has been based on a lack of knowledge of their essential nutritional needs. Evidence is presented in this paper that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood or blood derivatives for its growth requirements. It has been shown that this organism will grow in the total absence of blood derivatives, meat extractives, and animal peptones. The growth accessory substances which occur in blood have been found to exist also in plant tissue. Sterile raw potato can replace blood in the cultivation of *Bacillus influenzæ*.

On the basis of the facts presented, it seems not unreasonable to assume that nutritional deficiency in the cultivation of other bacteria may be overcome by the addition to culture media of the appropriate growth accessory substances.

CONCLUSIONS.

1. Growth of *Bacillus influenzæ* depends on two distinct and separable substances.
2. These growth accessory substances are present in blood.
3. They occur in plant as well as in animal tissue.
4. Sterile, raw potato will serve as a substitute for blood in the cultivation of *Bacillus influenzæ*.

BIBLIOGRAPHY.*

1. Thjötta, T., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 197.
2. Thjötta, T., *J. Exp. Med.*, 1921, xxxiii, 763.
3. Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.
4. Davis, D. J., *J. Infect. Dis.*, 1907, iv, 73.
5. Davis, D. J., *J. Infect. Dis.*, 1917, xxi, 392; 1918, xxiii, 248.
6. Ghon, A., and von Preyss, W., *Centr. Bakt., Ite Abt., Orig.*, 1902, xxxii, 96.
7. Olsen, O., *Centr. Bakt., Ite Abt., Orig.*, 1920, lxxxv, 12.
8. Fildes, P., *Brit. J. Exp. Path.*, 1921, ii, 16.
9. Falk, K. G., McGuire, G., and Blount, E., *J. Biol. Chem.*, 1919, xxxviii, 229.
10. Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145.
11. Wildiers, E., *La Cellule*, 1901, xviii, 313.
12. Bottomley, W. B., *Proc. Roy. Soc. London, Series B*, 1915–17, lxxxix, 102, 481.

---

* Since the acceptance of this paper for publication the following articles have appeared on the accessory substances concerned in the growth and symbiosis phenomenon of Pfeiffer's bacillus:

1. Rivers, T. M., and Poole, A. K., Growth requirements of influenza bacilli, *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 202.
2. Rivers, T. M., Growth of influenza bacilli without blood, *J. Am. Med. Assn.*, 1921, lxxvi, 1744.
3. Davis, D. J., Food accessory factors in bacterial growth. III. Further observations on the growth of Pfeiffer's bacillus (*B. influenzæ*), *J. Infect. Dis.*, 1921, xxix, 171; IV. The "satellite" or symbiosis phenomenon of Pfeiffer's bacillus (*B. influenzæ*) 178; V. The value of the satellite (or symbiosis) phenomenon for the classification of hemophilic bacteria, 187; VI. Further observations on the substances necessary for the growth of Pfeiffer's bacillus, *J. Am. Med. Assn.*, 1921, lxxvii, 683.

STUDIES ON THE PHENOMENON OF D'HÉRELLE WITH BACILLUS DYSENTERIÆ.

By MARTHA WOLLSTEIN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 35 to 39.

(Received for publication, May 31, 1921.)

The phenomenon of d'Hérelle (1) is the expression of a lytic reaction occurring between a bacterium which is inducing an infection in an animal and a substance elaborated in that organism, probably by the leucocytes and other tissue cells, in response to the stimulus of the metabolic products of the invading bacterium. The important element of the reaction is that bacteria exposed to the lytic substance acquire the ability to transfer the lytic property to subsequent generations. The first observations of d'Hérelle were made on stool filtrates from patients convalescent from bacillary dysentery (1). As a result of this and further studies, d'Hérelle (2) concluded that whenever an animal offers resistance to a pathogenic bacterium, a bacteriophage, active for that bacterium, can be isolated from the dejections of the animal. He believes that defense by bacteriophage is a specific immunity reaction. Dumas (3) was able to demonstrate that filtrates from specimens of Paris city water, of Seine water, and of earth, were lytic for colon and dysentery bacilli. He consequently expressed the opinion that the phenomenon is non-specific. The explanation of Dumas' results, as McLeod (4) has stated, is that the specimens he examined were contaminated with excreta infected with colon or dysentery bacilli or both. Specimens from other places may give negative results.

EXPERIMENTAL.

Intestinal Contents.

In an attempt to demonstrate d'Hérelle's phenomenon in human intestinal contents, twenty-three patients at the Babies' Hospital were examined. Six of the children gave a history of gastrointestinal disturbance, while one had been observed throughout an attack of Shiga bacillus dysentery during which the bacillus had been isolated from the stools. Specimens from twenty-two of the twenty-three patients, including the dysentery convalescent, when grown in broth for 24 hours and then filtered through a Berkefeld candle, showed only a negative (non-lytic) reaction between the sterile filtrate and young cultures of *Bacillus dysenteriæ*. One positive reaction was obtained with the filtrate from the feces of an infant who had died of peritonitis due to *Bacillus coli* after intussusception of 3 days duration. In this instance the lytic action was stronger on the colon bacillus than on the dysentery bacillus, and it was possible to transfer the lytic quality to later generations of *Bacillus coli*.

The negative results obtained in the case of the infant who had recovered from an attack of Shiga dysentery may mean that the lytic quality of the intestinal contents is of short duration after such an infection. That the phenomenon of d'Hérelle is not a widespread one among children not ill of gastrointestinal disease is quite evidently demonstrated by these observations.

Dumas' (3) positive lytic reaction with the stools of five of eight adults who gave no history of previous intestinal infection would seem to show that there may be a difference between children and adults in this respect.

Guinea Pig Peritoneal Exudate.

Bordet (5) interpreted the lytic phenomenon of d'Hérelle as due to leucocytic action. He demonstrated its presence in the peritoneal exudate of guinea pigs inoculated with *Bacillus coli* on three occasions at intervals of 5 days. Repeated attempts to reproduce Bordet's results were negative, until a guinea pig was killed after only one intraperitoneal injection; that is, before the peritoneal exudate of the animal had become antilytic by repeated inoculations (Bordet).

The peritoneal exudate is withdrawn by means of sterile capillary tubes from the living animal, and diluted with twice its quantity of sterile broth adjusted to a pH of 8. This is in accordance with Gratia's (6) observation that the lytic action of d'Hérelle proceeds more readily and completely in an alkaline than in an acid medium. In order to rid the guinea pig exudate of fibrin it is well to draw it into a tube containing small sterile beads. The tube containing the defibrinated exudate is sealed and kept for 3 to 30 days to complete the leucocytic action (Bordet). At the end of that time it is centrifuged and the clear supernatant fluid heated for ½ hour at 58°C., after which it is ready to be tested on broth or agar slant cultures. Both must be young, not over 2 to 3 hours old. 1 drop of the fluid to be tested is dropped along one side of an inoculated agar slope, and the tube set upright in the thermostat for observation during the next 48 hours. To broth cultures a larger quantity of the fluid must at first be added, but after one or two generations the lytic fluid may be found to be active in high dilutions.

Growth may be prevented in a broth culture if the lytic agent be added when the tube is inoculated, or young bacilli may be dissolved if the fluid be added after the culture has grown 2 to 4 hours. A broth culture to which the lytic fluid has been added remains or becomes more or less clear for 12 to 24 hours, then it may become somewhat turbid, and clear again after another day. Even the clear tubes are not always sterile, as can be shown by plating. On agar slants the action of the lytic fluid is evidenced by a clear streak along the track of the fluid (Fig. 1). In this clear area there may be no colonies, or there may be a few; that is, some resistant bacilli may remain undissolved and viable. The action of the lytic fluid serves to separate a given culture into sensitive and resistant strains, and by repeating the action on the resistant strains a more and most resistant one can be obtained.

By filtering the clear fluid resulting from the action of the original lytic filtrate or exudate on a broth culture, any quantity of lytic fluid can be obtained, and its action on any number and variety of organisms tested.

The bacteriophage obtained by inoculating a strain of Shiga dysentery bacilli into a guinea pig was found to dissolve its homologous strain, five other Shiga bacillus strains, and two Flexner dysentery bacillus strains. On three strains of Hiss Y bacilli, however, its action was less marked; while three strains of colon bacilli, and one strain

each of typhoid, paratyphoid A and B, mouse typhoid, Morgan bacillus, hog-cholera, and fowl-cholera were not attacked.

Dr. Gratia kindly gave me a small quantity of a lytic fluid which he had prepared by allowing guinea pig exudate, obtained by Professor Bordet in Brussels, to act on a resistant strain of *Bacillus coli*. This bacteriophage proved to be strongly lytic for strains of *Bacillus dysenteriæ* Shiga and less lytic for two strains of Flexner and three strains of Hiss Y dysentery bacilli. The Brussels strain of *Bacillus coli* as well as a strain isolated at the Babies' Hospital some months ago were readily dissolved, but a recently isolated strain of colon bacilli was not affected. *Bacillus typhosus* was acted upon to a slight extent only, but neither a paratyphoid A nor B strain was attacked.

These results showed that the lytic power of the colon phage, made with a resistant *Bacillus coli* strain, is not specific, but that it acts in varying degree on the related strains of the colon-typhoid-dysentery groups of bacilli. The observations of Bordet and Ciuca (7) announcing this fact were published just as our experiments were completed.

One strain each of Friedländer's bacillus, *Bacillus choleræ suis*, *Bacillus avisepticus*, *Bacillus typhi murium*, and Morgan's bacillus was tested with negative results.

With the purpose of studying more fully the effect of the action of a bacteriophage on the dysentery bacillus, a strain of the Shiga type was selected and exposed to the action of the colon phage. A new bacteriophage resulted which was strongly lytic for all strains of dysentery bacilli, for typhoid bacilli, and for the Belgian and American strains of *Bacillus coli*. The paratyphoid strains were still unaffected.

The anti-colon bacillus bacteriophage showed certain group reactions on members of the dysentery-colon-typhoid group. It was most active on the colon strain with which it had been made, less active on a heterologous colon strain; equally active on Shiga dysentery strains, less so on Flexner strains, and still less so on Hiss Y strains; and it was only slightly active on a strain of typhoid bacilli. The Shiga antidysentery bacillus phage, on the other hand, was completely lytic on all strains of Shiga dysentery bacilli and only slightly less so on all strains of Flexner and Hiss Y strains. It was completely lytic for Belgian and American strains of *Bacillus coli* and decidedly lytic for a typhoid bacillus strain. Its group reactions

were stronger than those of the colon bacillus phage. The question naturally presented itself as to whether this denotes a stronger bond between typhoid and dysentery bacilli than between dysentery and colon bacilli, or whether the lytic power of the dysentery bacteriophage was fundamentally stronger for all strains of these three groups. Weight is given to the former supposition by the fact that while the colon phage acted very slightly on the typhoid bacillus, a typhoid phage made from the colon phage by exposure to a strain of *Bacillus typhosus* acted much more strongly on dysentery than on *coli* strains.

Further study of the effect of the action of a lytic fluid upon a strain of Shiga dysentery bacilli is of interest. Not all the bacilli are dissolved, and it follows that those which survive are resistant to the lytic principle. Plates poured from a fluid culture of Shiga dysentery bacilli after 24 or 48 hours contact with a lytic fluid, when the broth culture tube appears to be perfectly clear, show two kinds of colonies (Figs. 2 to 4). One is round, with regular edges, a heaped up or denser center, and rather moist growth. The second kind is very irregular in size and shape, with deep indentations in its thin edges, a depressed center, and very granular. In broth the regular colonies grow rapidly and profusely, the broth becoming turbid. The irregular colonies grow more slowly and fall to the bottom of the tube in granular masses, leaving the broth slightly turbid or almost clear.

A strain of Flexner dysentery bacilli exposed to dysentery bacillus bacteriophage gave rise to regular and irregular colonies exactly comparable to those obtained from the acid or Shiga dysentery strains. Kuttner (8) has shown that the typhoid bacillus develops two kinds of colonies after action of a typhoid bacteriophage.

The experiment presented in Table I was done in order to test the reaction of the bacilli in the two kinds of colonies to the lytic fluid. The table shows that the bacilli in the regular colonies are more resistant to the lytic principle than are those in the irregular colonies.

To ascertain which colonies carry the lytic agent, *i.e.* which are lysogenic, the experiment presented in Table II was done. The irregular colonies are found, then, to be lysogenic and sensitive, while the regular colonies are non-lysogenic and resistant.

Both the regular and the irregular colonies were subcultured on plates for more than 40 generations, and Figs. 5 and 6 show the results. The regular colonies retain their regular outline and characteristics. The irregular colonies, on the other hand, gradually lose their markedly irregular outline and tend to approximate more nearly the regular type of colony, until there are more regular than irregular ones present, and even these are less radically irregular. That is, while the edges of the irregular colonies may still be deeply serrated, the centers are

TABLE I.

Shiga Dysentery Bacilli Plus Shiga Bacteriophage.

Strain.	Medium.	Age.	Result.	
			After 24 hrs.	After 48 hrs.
		hrs.		
Normal, or original.....	Broth.	2	Lysis; tube clear.	Same.
Regular.....	"	2	No lysis; tube turbid.	"
Irregular.....	"	2	Lysis; tube clear.	"

TABLE II.

Normal or Original Strain of Shiga Dysentery Bacillus.

Phage.	Medium.	Age.	Result.
		hrs.	
Normal, original bacillus filtrate.....	Broth.	2	Lysis.
Regular colony filtrate.....	"	2	No lysis.
Irregular " "	"	2	Complete lysis.

less hollowed out and gradually become dense (Figs. 7 and 8). The explanation seems to be that the sensitive bacilli die off more rapidly than the resistant ones, which form the regular colonies in later generations. It is a matter of selection.

The two kinds of dysentery bacilli, resistant and sensitive, do not differ from the original culture in their sugar reactions.

An attempt was made to determine whether the acquired lysogenic property is retained permanently or lost in later generations. Working with the original, normal culture as a whole, it was found that

after the seventh generation on agar or in broth the bacilli which had survived contact with a lytic fluid were no longer able to transmit the lysogenic property to other cultures or to dissolve the normal Shiga bacilli. Apparently the sensitive, lysogenic bacilli had all been eliminated in seven generations.

The experiments on this point were repeated with bacilli from colonies isolated from a normal Shiga culture which had been exposed to dysentery bacteriophage and then plated. The resulting, undissolved bacilli must all be resistant to some degree, but the colonies which grew were of the two distinct types noted above, and were designated sensitive resistant, or irregular, and resistant resistant, or regular. The bacilli from the regular colonies were not dissolved by the dysentery bacteriophage, nor did their broth filtrate dissolve normal Shiga bacilli of the original strain, or the bacilli of the irregular colonies. On bacilli from the regular colonies some lysis resulted. The bacilli from the irregular colonies, on the other hand, were dissolved by the dysentery phage and by their homologous filtrate, which also dissolved normal dysentery bacilli and acted to some extent on the bacilli of the regular type of colony. This was explained by the assumption that the culture of regular, resistant bacilli was not absolutely pure in the sense that some less resistant bacilli were still present. By plating, this was proved to be correct, for both regular and irregular colonies developed from a broth tube of regular bacilli which had been in contact with lytic fluid over night. The regular colonies fished from this plate were again subjected to the action of the strong phage, and plated after 4 days. The resulting colonies were all of the regular type, and were not dissolved by the strong dysentery phage or by filtrates from regular or irregular types of bacilli. It is possible then to obtain a pure, resistant type of dysentery bacilli by repeated elimination of sensitive bacilli from the strain, these resistant bacilli having been present originally.

The sensitive type of dysentery bacillus, after nine generations of broth and plate subculturing, was found to have become more resistant, as shown by the fact that the bacilli of the ninth generation were acted upon less strongly by the antidysenteric phage and by the filtrate from the first generation of irregular bacilli. The filtrate

from the ninth generation was also less lysogenic than the filtrate from the first generation had been.

The sensitive bacilli gradually die out by the action of the lytic substance they carry, and gradually resistant bacilli only are left. The tendency then is for cultures of sensitive bacilli to become more resistant and for resistant bacilli to remain resistant. This is also demonstrated by the colonies on repeated subcultures (Figs. 5 and 6).

The Lytic Reaction under Anaerobic Conditions.

The lytic action proceeds as rapidly and as completely in the absence of oxygen as in its presence. Deep broth cultures with rabbit kidney and sealed with vaseline were inoculated with dysentery bacilli and antidysenteric phage, and growth was prevented as it was in the aerobic controls.

Morphology.

The sensitive strains are composed of short bacilli and many coccoid and round, swollen forms. The resistant strains are made up of regular, equal sized, and evenly staining bacilli, with some larger forms, but few, if any, coccoid or swollen, round forms. Threads are seen in older cultures of the more resistant types.

Virulence.

Rabbits inoculated with minute doses of early broth cultures of the resistant bacilli became paralyzed within 48 hours, and did not recover. Cultures of the sensitive bacilli required five times as great a dose to produce similar results. Apparently the exotoxin of the resistant type of dysentery bacilli is much stronger than that of the sensitive type.

Immunization.

Rabbits were immunized with resistant strains of Shiga dysentery bacilli, with normal strains, and with Shiga bacteriophage. Agglutination tests with the resulting sera brought out the fact that normal, irregular or sensitive, and regular or resistant strains are agglutinated well, the resistant somewhat less so than the sensitive. But a highly resistant strain was not agglutinated even by the sera made with

ordinarily resistant strains. The highly resistant strain killed the animals before they developed immune bodies in their sera.

The animals immunized with antidysenteric phage agglutinated normal dysentery strains in dilution of 1:200, sensitive strains in 1:1,000, and resistant strains in 1:100, while the highly resistant strain was not agglutinated.

Evidence which shows that d'Hérelle's phenomenon goes on in the human intestine in a patient with dysentery was recently obtained. At autopsy on an infant 9 months old, who died of bronchopneumonia, a severe pseudomembranous enterocolitis was found. The child had only been under observation for 48 hours, but a history of intestinal symptoms was obtained. By proctoscopic examination on the day before death ulcers were seen in the rectum and a specimen of feces was obtained and plated. On the plates Flexner dysentery bacillus colonies developed with marked, irregular, indented edges. A few small, regular colonies were also noted. Fishings from both kinds of colonies were made, and a sensitive and a resistant strain was obtained. The plates made directly from the rectal specimen were very similar to those obtained in our experiments on the action of the antidysentery lytic fluid on dysentery bacilli. Whether, if a complicating pneumonia had not intervened, the child would have recovered from the dysentery infection or whether the resistant dysentery bacilli were present in sufficient numbers to prevent recovery in spite of the active lysis going on, remains a question.

SUMMARY.

It has been shown that a lytic fluid for dysentery bacilli can be obtained from the peritoneum of the guinea pig by intraperitoneal inoculation of live dysentery bacilli, and that the lytic action of such a fluid is not strictly specific, but that it exerts a group action on the dysentery-colon-typhoid group of bacilli. A lytic fluid with similar effects was obtained from a child dying of Flexner dysentery infection, and an anti-colon bacillus lytic fluid from a child who died of intussusception with colon bacillus peritonitis.

The action of the lytic fluid on the dysentery bacilli, both *in vivo* and *in vitro*, is to divide the culture into sensitive and resistant strains, and the latter can be carried to a degree of very marked, if not complete resistance to lysis. Such resistant strains are not lysogenic, nor are

they agglutinable. The sensitive strains are lysogenic and agglutinable. Varying degrees of sensitive and resistant bacilli exist in a single culture. The sensitive bacilli gradually lose the lysogenic property which they acquired under special conditions, but the very resistant bacilli never acquire that property. It is conceivable that the resistant strains are responsible for the untoward outcome of disease in human beings. Theoretically the administration of lytic fluid should rid the intestinal tract of most of the infecting bacilli, and only if completely resistant bacilli in large numbers remain unacted on is the outcome of the disease a fatal one.

BIBLIOGRAPHY.

1. d'Hérelle, F., *Compt. rend. Acad.*, 1917, clxv, 373.
2. d'Hérelle, F., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 538; *Compt. rend. Acad.*, 1921, clxxii, 99.
3. Dumas, J., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1314.
4. McLeod, J. W., and Govenlock, P., *Lancet*, 1921, i, 900.
5. Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1293.
6. Gratia, A., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 275.
7. Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 278.
8. Kuttner, A., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 158.

EXPLANATION OF PLATES.

PLATE 35.

FIG. 1. Effect of the bacteriophage on an agar slope culture of *B. dysenteriæ*.

FIG. 2. Agar plate of a culture of *B. dysenteriæ*.

PLATE 36.

FIG. 3. Regular colonies of *B. dysenteriæ* after the action of the bacteriophage on a normal strain.

FIG. 4. Irregular colonies of *B. dysenteriæ* after the action of the bacteriophage on a normal strain.

PLATE 37.

FIG. 5. Regular colonies of *B. dysenteriæ* after 40 generations of growth on agar plates.

FIG. 6. Irregular colonies of *B. dysenteriæ* after 40 generations of growth on agar plates.

PLATE 38.

FIG. 7. Irregular colonies of *B. dysenteriæ;* third generation; thin edges and hollowed centers.

PLATE 39.

FIG. 8. (*a*) Regular colonies of *B. dysenteriæ;* 40th generation. (*b*) Irregular colonies; 40th generation; irregular edge and heaped up center.

FIG. 1.

FIG. 2.

• (Wollstein: Phenomenon of d'Hérelle.)

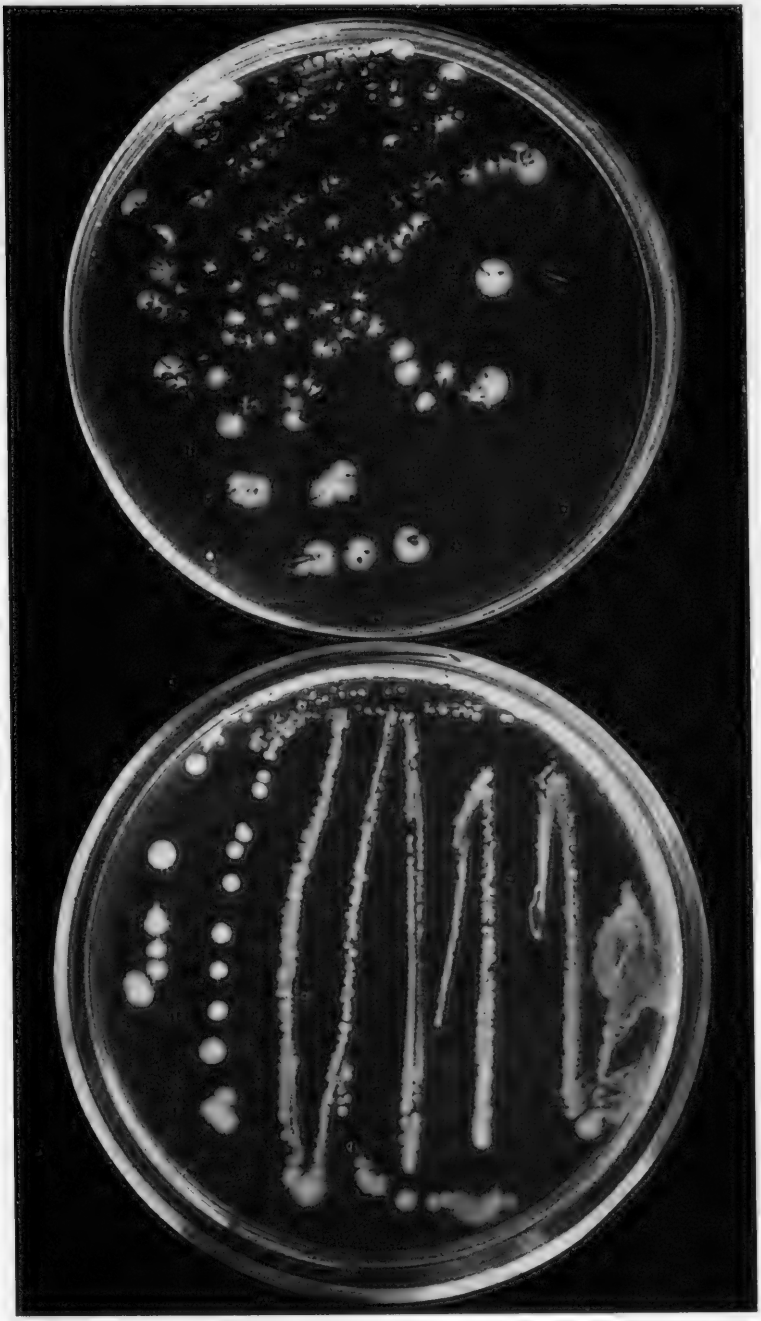


FIG. 4.

FIG. 3.

(Wollstein: Phenomenon of d'Hérelle.)

FIG. 6.

FIG. 5.

(Wollstein: Phenomenon of d'Hérelle.)

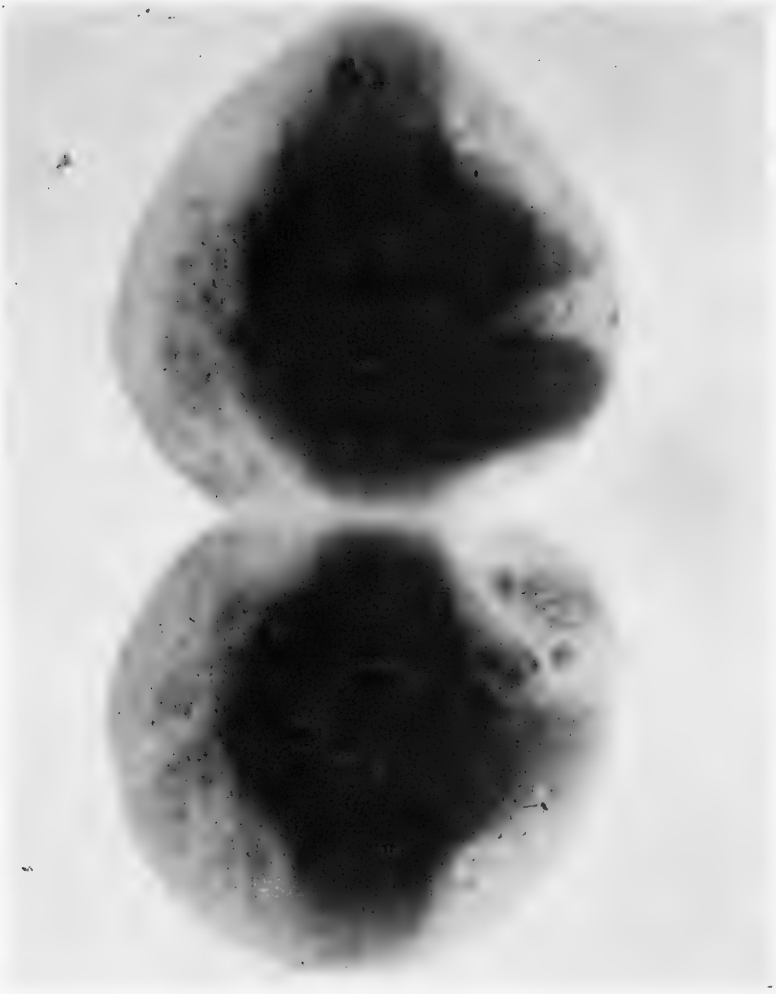


FIG. 7.

(Wollstein: Phenomenon of d'Hérelle.)

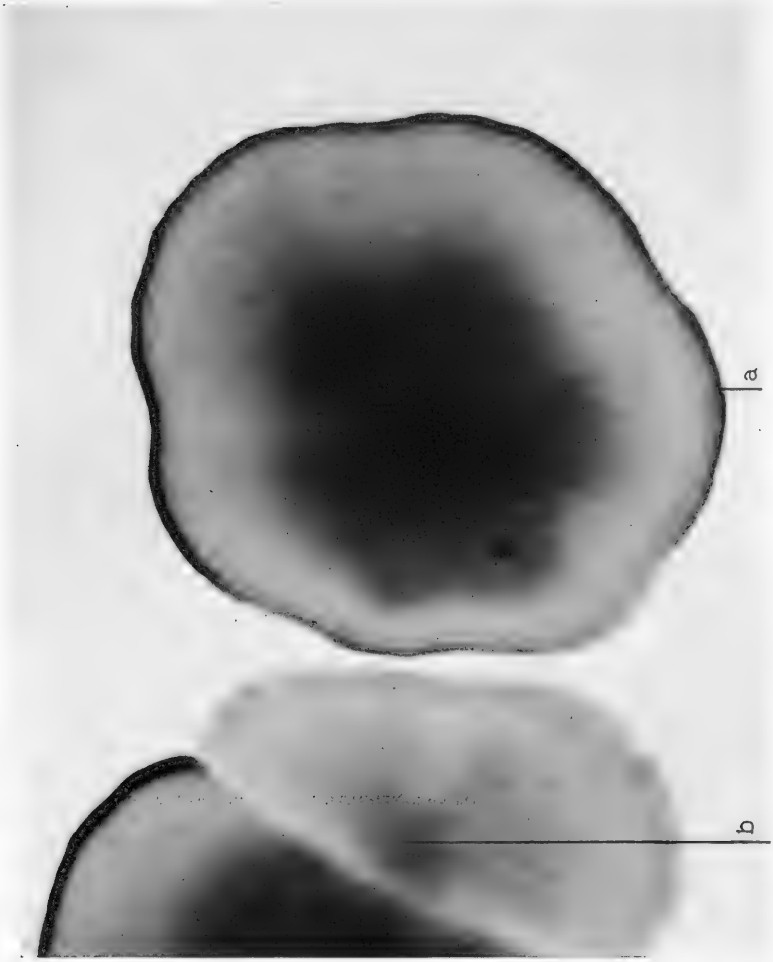


FIG. 8.

(Wollstein: Phenomenon of d'Hérelle.)

THE BIOLOGICAL AND THE SEROLOGICAL REACTIONS OF INFLUENZA BACILLI PRODUCING MENINGITIS.

By T. M. RIVERS, M.D., and LAWRENCE A. KOHN.

(From the Department of Pathology and Bacteriology of the Johns Hopkins University, Baltimore.)

(Received for publication, June 7, 1921.)

Influenzal meningitis has been drawn into the controversy over the etiology of influenza, first by one faction and then by another, without much justification or proof by either. The relation of influenza bacilli to the disease influenza may not be settled for a long time and certainly not before much more is known about the biological and the serological behavior of these organisms. The study of influenza bacilli from the throats, sputum, and lungs of individuals with and without influenza has led to numerous opinions about the unity or multiplicity of strains of these bacilli. Even different competent workers (1) using the same strains do not obtain similar results. There must be some cause, which perhaps has not yet been discovered, to account for all these discrepancies. Since there are so many opinions about the relation of *Bacillus influenzæ* to influenza and about the relation of one influenza bacillus to another, it was considered wise to select first for study a small group of the organisms which were known positively to be pathogenic. Influenza bacilli which had produced meningitis offered the group sought.

No attempt will be made at this time to cover the various ideas about influenza bacilli in general. The work of only a few investigators will be cited to show the multiplicity of opinions even about *B. influenzæ* producing meningitis. Slawyk (2) isolated an influenza bacillus from the blood and spinal fluid of a child with meningitis. This organism was seen by Pfeiffer, who agreed that it was *B. influenzæ*. Cohen (3) recovered from the spinal fluid and blood of patients with meningitis an organism similar to the influenza bacillus, except that it caused septicemia in rabbits, and because of this difference in virulence and evidence obtained by protection experiments, he believed the two organisms were not the same. Wollstein (4, 5) in her studies on influenzal meningitis concluded that

respiratory and meningeal strains of *B. influenzæ* were more or less identical culturally and immunologically, varying only in virulence. Davis (6) found no difference in the virulence of respiratory and meningeal strains. Anderson and Schultz (7) isolated five strains of influenza bacilli from one patient with meningitis, one each from the spinal fluid, blood, nose, nasopharynx, and throat, and reported them to be serologically different. This is a rather unusual finding in view of Wollstein's (4) work, who showed that *B. influenzæ* injected intravenously into rabbits and subdurally into monkeys could be recovered from the mucous membranes of the upper respiratory tract.

For a long time influenza bacilli were considered alike culturally, but lately many have shown that they can be subdivided by biological reactions. Wadsworth and Wheeler (8), Jordan (9), Stillman and Bourn (10), Rivers (11), and Rivers and Leuschner (12) have contributed to this work. The bacilli also were generally accepted as being hemoglobinophilic or hemophilic, but now there is good reason to believe that they are not hemoglobinophilic (13–16), and possibly not even hemophilic (17). At any rate the strains studied in this work meet all the old criteria of influenza bacilli—they are Gram-negative, non-motile, aerobic, pleomorphic bacilli which do not grow on ordinary nutrient or meat infusion agar when in pure culture.

Thirteen strains of *Bacillus influenzæ* isolated from spinal fluids of patients who died of influenzal meningitis were used. Seven of these (Nos. 7, 8, 9, 10, 11, 12, and 16) were furnished by Dr. A. W. Williams and were from a collection made by the Department of Health of the City of New York during a period of 7 years, 1913–20. Povitzky and Denny (18) have already published work on these seven strains. The fact that the results presented here do not coincide entirely with theirs places the authors under no less of an obligation to the New York workers for supplying most of the strains studied. Strain 16 was obtained from Dr. Wollstein and may be the same as Strain 6 obtained directly from Dr. Wollstein by this laboratory. Proof, however, that these two strains came from the same patient is lacking. In this work these have been considered as separate strains. No. 17 was recently furnished by Dr. E. O. Jordan who received it from Dr. D. J. Davis, of the University of Illinois, in November, 1920. Strains 1, 2, 14, and 15 were isolated here from patients in Dr. John Howland's clinic. For comparison four blood culture strains from children with pneumonia were used. No. 3 was obtained in pure culture from a child who later recovered; Strains 4 and 5, mixed with Type IV pneumococci, and No. 13 were obtained at autopsies. A list of the strains is given in Table I.

TABLE I.

List of Strains.

Strain No.	Label when received.	Source.	Date of isolation.	From whom received.
1		Meninges; influenzal meningitis.	1920	Rivers.
2		" " "	1920	"
3		Antemortem blood culture; pneumonia.	1920	"
4		Postmortem blood culture; pneumonia.	1920	"
5		Postmortem blood culture; pneumonia.	1920	"
6	B. inf. Men. 1917	Meninges; influenzal meningitis.	1917	Wollstein.
7	Z	" " "	1913–14	Williams.
8	747	" " "	1913–14	"
9	6741	" " "	1918	"
10	6746	" " "	1918	"
11	6827	" " "	1919	"
12	6745	" " "	1918	"
13		Postmortem blood culture; pneumonia.	1920	Rivers.
14		Meninges; influenzal meningitis.	1920	"
15		" " "	1919	"
16	B. I. W.	" " "	1917(?)	Williams.
17	Pfeiffer No. 26	" " "	Received by Dr. Jordan 1920.	Jordan.

Biological Reactions.

One particular kind of medium was used so much in the study of cultural characteristics and serological reactions that it will be described in detail and hereafter will be spoken of as hemo-peptone water for convenience.

Peptone (Fairchild's) ..... 20 gm.
Sodium chloride ..... 5 gm.
Distilled water ..... 1,000 cc.

Boiled and titrated to pH 7.4.

10 cc. of washed red blood cells added and heated to 95°C.

Filtered through paper.

Sterilized through a Mandler filter. Tubed or flasked in required amounts and incubated for sterility.

This medium is perfectly clear, contains very little protein as it will not cloud on boiling or autoclaving, and supports a splendid growth of influenza bacilli.

Production of Indole.—Tubes of hemo-peptone water, after inoculation and incubation for 3 to 10 days, were extracted with ether. This ether extract was decanted and tested with Ehrlich's reagent for indole. All the meningitic strains except No. 10 gave a positive test, while this was true of only two (Nos. 3 and 13) of the four blood culture strains. No. 16, which Povitzky and Denny (18) said failed to produce indole, did so about three-fourths of the time when hemo-peptone water alone was used, and all the time when tryptic digest of milk was added to the hemo-peptone water. No. 10 in the meningitic group and Nos. 4 and 5 from the blood culture group never under any conditions produced indole.

Reduction of Nitrates.—Hemo-peptone water plus potassium nitrate was inoculated, incubated 5 days, and then tested with the sulfanilic acid and naphthylamine reagent for nitrites. All the strains regularly reduced nitrates. In 1920, Rivers (11) stated that some influenza bacilli did and others did not reduce nitrates to nitrites. At that time he suggested that further work might show nitrite formation to be a common characteristic of these bacilli. Meat infusion broth, blood, and potassium nitrate were the medium used then. On this medium nitrate reduction takes place irregularly. If hemo-peptone water plus potassium nitrate be used, however, nitrites are formed regularly by all influenza bacilli so far tested.

Hemolysis.—None of these strains hemolyze red blood cells either in solid or in liquid media.

Production of Amylase.—A starch-splitting ferment is not produced by any of these strains.

Fermentation of Sugars.—For many years influenza bacilli were considered unable to ferment any of the sugars. Lately a number of American (8, 10, 18) and German (19, 20) workers have reported positive fermentations with various kinds of media and sugars. Stillman and Bourn (10) have done the best work and have reported many of the difficulties encountered in fermenting sugars with these bacilli.

In 1920, Rivers (11) stated that he could get no positive fermentations. At that time meat infusion broth plus fresh rabbit blood was used as the medium to which the test sugars were added. Since then it has been found that this is a very unsatisfactory way to test sugar fermentations, because quite often there was no evidence of the fermentation of glucose. At other times when acid was formed the reaction was more strongly marked in the control tubes to which no sugar had been added. Obviously this was an unsatisfactory method if any dependable results were to be obtained. A more suitable basic medium was sought to which the sugars could be added. The hemopeptone water described above was used with some success, but even with this medium the results were not so regular as desired.

The following medium was then tried.

Peptone (Fairchild's)	20 gm.
Sodium chloride	5 gm.
Agar-agar	15 gm.
Distilled water	1,000 cc.

This was boiled, titrated to pH 7.4, filtered, and autoclaved in 100 cc. quantities. After it was taken from the autoclave and while it was still at 95°C., 1 cc. of washed red blood cells, 10 cc. of a 10 per cent solution of the required sugars, and enough 25 per cent alcoholic solution of brom-cresol purple to give a good color, were added. While still warm the medium was tubed and later incubated for sterility. It is desirable to make the medium so that there is a good deal water of condensation. The coagulated blood cells, it is true, give the medium a dirty appearance but do not interfere with the results. Later a clear medium was made by adding to the peptone agar 15 to 20 cc. of an autoclaved blood clot extract and 15 to 20 cc. of a filter-sterilized yeast extract. When glucose and brom-cresol purple were added to this clear agar a better looking medium was the result and fermentation of the sugar took place. Eventually some kind of clear medium such as that just described may be used, but the findings in this paper are based on the results obtained upon the muddy looking solid medium. Readings were made daily for 10 days.

Sugar fermentations were more prompt and regular when the solid medium was used instead of the hemo-peptone water; for example, none of the strains fermented filter-sterilized maltose in a liquid medium, while Strain 4 always fermented it in the solid medium. (Autoclaved maltose gave very irregular results, probably because it is so easily hydrolyzed.) The fructose tubes were distinctly acid after 48 hours, but later often returned to about the same reaction as that of the controls. The reaction of some of the unfermentable sugar media, lactose and rhamnose for example, at times was made distinctly more alkaline by the growth of these bacilli. Apparently influenza bacilli under the proper conditions may form both acid and alkali and the final reaction is the resultant of these two factors.

Nineteen so called sugars have been tested. Sucrose, lactose, mannitol, mannose, dulcitol, dextrin, glycerol, rhamnose, inosite, sorbitol, salicin, and inulin were not fermented by any of these strains. Acid without gas was produced by all, except No. 17, from glucose, fructose, and galactose (Table II). Upon raffinose and arabinose questionable results were obtained. All except Nos. 13 and 17 formed acid without gas from xylose. Only No. 4, a blood culture strain, fermented maltose. At a glance it can be seen that the meningitic strains with the exception of No. 17 and two of the blood culture strains gave similar fermentation reactions. Strain 17 fermented none of the sugars.

Action on Milk and Milk-Broth.—In 1920, Rivers (11) stated that a certain group of influenza bacilli, which contained five meningitic strains, made milk-blood-broth slightly acid in 48 hours while others made it slightly alkaline. The production of acid or alkali in milk-broth mixtures by the organisms here studied varied very much when different lots of the medium were used. At times acid was formed, at others alkali. Usually all of them acted similarly upon the same medium. This variation depended most likely on the amount of glucose in the broth used, as the same variation was noted on blood-broth mixtures alone. Dochez[1] has found that the glucose in different lots of meat infusion broth varies considerably, as shown by quantitative determinations. When milk with heated washed red blood cells alone was used, however, all the strains fairly regularly produced a slight amount of alkali. The use of milk-blood-broth seems inad-

[1] Dochez, A. R., personal communication.

visable as a routine procedure. If milk is to be used at all, skimmed milk without broth should be chosen, as the results will be more constant and reliable.

TABLE II.

Cultural Characteristics.

Strain No.	Source.	Indole production.	Reduction of nitrates.	Glucose.	Fructose.	Galactose.	Maltose.	Xylose.	Milk-broth.	Milk.
1	Meninges; influenzal meningitis.	+	+	+	+	+	—	+	?	Alkali.
2	" "	+	+	+	+	+	—	+	?	"
6	" "	+	+	+	+	+	—	+	?	"
7	" "	+	+	+	+	+	—	+	?	"
8	" "	+	+	+	+	+	—	+	?	"
9	" "	+	+	+	+	+	—	+	?	"
10	" "	—	+	+	+	+	—	+	?	"
11	" "	+	+	+	+	+	—	+	?	"
12	" "	+	+	+	+	+	—	+	?	"
14	" "	+	+	+	+	+	—	+	?	"
15	" "	+	+	+	+	+	—	+	?	"
16	" "	+	+	+	+	+	—	+	?	"
17	" "	+	+	—	—	—	—	—	0	0
3	Blood culture; pneumonia.	+	+	+	+	+	—	+	?	Alkali.
4	" " "	—	+	+	+	+	+	+	?	"
5	" " "	—	+	+	+	+	—	+	?	"
13	" " "	+	+	+	+	+	—	—	?	"

The most important positive cultural characteristics are given in this table. Under the sugars + indicates acid production without gas, — indicates production of neither acid nor gas. Under milk-broth ? indicates that the reaction sometimes became more acid, at other times more alkaline, 0 indicates that the test was not made.

Serological Reactions.

For the study of serological relations the agglutination reaction was chosen, chiefly because it can be verified and extended by the absorption of agglutinin test. Moreover, in the majority of the recent work on influenza bacilli this reaction has been used and it was felt that whatever correlation obtained with meningitic strains would stand out all the more sharply from the heterogeneity of respiratory strains.

Preparation of Immune Sera.—For the study of agglutinations and absorptions monovalent antisera were prepared from rabbits with Strains 1, 3, 4, 5, 6, 8, 10, 11, 13, 14, and 15, and from sheep with Strain 9. Preliminary bleedings were titered for normal agglutinins. Most of the normal sera agglutinated none of the various strains in a greater dilution than 1:20. Several, however, agglutinated a few of the strains in a dilution of 1:40. In subsequent work normal agglutinins did not interfere as 1:100 was the first dilution in each series.

The technique of immunizing the rabbits consisted in intravenous inoculation of live 24 hour cultures in cooked rabbit blood meat infusion broth, beginning with 0.25 cc. and gradually increasing the dose up to 1 to 1.5 cc. Three series of injections were made on 3 successive days. Each series was followed by a rest of 4 days. 5 days after the last inoculation test bleedings were titered for agglutinins. The animals whose sera showed a titer of 1:1,200 or over were bled and the serum was collected and preserved with 0.5 per cent phenol. Many sera had a titer of 1:3,000 or over. Animals whose sera were unsatisfactory and in some cases animals surviving the bleeding were given more inoculations. The sheep was immunized by giving intravenous inoculations of live cultures of Strain 9 in hemo-peptone water made with sheep blood. The initial dose of 0.5 cc. was gradually increased to 20 cc. Injections of more than 20 cc. of the broth cultures were not well tolerated. Then the bacteria were collected by centrifuging, and the largest dose given was bacteria from 100 cc. of broth culture suspended in 10 cc. of salt solution. The injections were made daily for 7 days, followed by a rest of 5 to 7 days.

Preparation of Antigens for Agglutination.—A few preliminary experiments showed that the hemo-peptone water would be suitable for the preparation of agglutinating antigens and that formalinization favored the agglutinability of a suspension. The medium was prepared in large quantities; 200 to 300 cc. were placed in 500 cc. Erlenmeyer flasks, and incubated several days to insure sterility. The organisms from which it was desired to prepare antigens were cultivated through eight to ten daily transplants in tubes of this medium, in the expectation, as Dreyer and his coworkers (21) found for organisms of the enteric group, that there would be a loss of the tendency to spon-

taneous agglutination with an increased sensitivity to agglutination by immune sera. This was found to be true with influenza bacilli. After a number of transplants a diffuse growth was obtained in 12 to 18 hours. Then 1 cc. of these rapidly growing young cultures was inoculated into the flasks of the medium and incubated for 6 to 24 hours at 37°C. When the growth was considered sufficient, but in no case later than 24 hours after inoculation, the cultures were removed from the incubator and 0.1 per cent of commercial formalin was added. These flasks were stored in the refrigerator, being removed for daily shaking. After 7 to 10 days the antigens were found to be suitable for use. They were compared in density with a certain antigen arbitrarily chosen as a standard and diluted with sterile saline solution containing 0.1 per cent formalin. The resultant opacity was approximately that of the Oxford standard cultures.

Formalinized antigens prepared in this manner were used throughout the serological work. They proved very agglutinable, not prone to spontaneous agglutination, and, it was felt, comparable from day to day, though successive antigens from the same culture varied somewhat in sensitivity, and several of the strains always gave far more flocculable suspensions than others.

After standing 3 to 4 weeks, several of the cultures, especially those of non-meningitic origin, suddenly began to flocculate spontaneously. This phenomenon was looked for after holding an antigen for a month. Often, however, the antigens remained satisfactory for much longer periods.

Agglutination Tests.—With sera and antigens prepared as described a series of agglutination tests was performed. The incubation period for the test was invariably 6 hours at 55°C. in the water bath. Readings were made with the naked eye and artificial light 15 minutes after removing the tubes from the bath. The results of these tests are shown in Table III.

It is apparent (1) that with the exception of one or two doubtful results the four non-meningitic strains are agglutinatively distinct from the group, and from each other; (2) that Strain 10 is agglutinated by practically no serum save its own, which in turn agglutinates only Nos. 8 and 15; (3) that Strain 17 is not agglutinated to titer by any of the sera used; (4) that the majority of the other strains are

TABLE III.

Results of Agglutination Tests.

Strain No.	Serum No.											
	1	6	11	15	8	9	14	10	3	4	5	13
1	++++	++++	+++	++++	++++	++	—	—	—	—	—	+
2	++++	++++	++++	+++	+++	+	—	+	+	—	—	+
6	++++	++++	+++	++	++++	+++	++	—	—	—	—	++
7	++++	++++	++++	++	++++	++++	+++	+	+	+	+	+++
11	++++	++++	++++	+++	++++	++++	—	+	—	—	—	++
12	++++	++++	++	+	+++	++	—	—	—	—	—	—
16	++++	++++	++++	++	++++	++++	+++	+	+	+	+	++
15	++	+++	++++	++++	+++	++++	++++	++++	—	—	+	++
8	++	+++	+++	+++	++++	++++	++++	++++	—	—	+	+
9	—	+++	+++	+++	++++	++++	+++	+	—	—	—	++
14	++	++	+++	++	++++	+	++++	—	+	—	—	++
10	—	++	—	—	+	—	—	++++	—	++	—	—
17	0	+	0	0	+	0	0	0	++	0	0	+
3	—	—	—	—	—	—	+	—	++++	—	—	—
4	—	—	—	—	—	—	—	+	—	++++	—	—
5	—	++	+	+++	+	—	+	+	—	—	++++	++++
13	—	+	—	+++	—	—	—	+	+	—	—	—

++++ indicates to titer or over; +++ three-quarter titer; ++ one-half titer; + any agglutination up to one-quarter titer; — no agglutination; 0 no test made. Note the marked cross-agglutination among meningitic strains. Nos. 3, 4, 5, and 13 are blood culture strains.

agglutinated by almost all the sera. Closer inspection, however, shows that Serum 1 picks out Nos. 2, 6, 7, 11, 12, and 16, and that Serum 14 agglutinates Nos. 7, 9, 8, 15, and 16, leaving Nos. 1, 2, 6, 11, and 12 less affected.

This marked degree of cross-agglutination emphasized the necessity of absorptions. Accordingly, these were begun at once without extensive repetition of agglutinations. It may be said at this point that the control agglutinations made with unabsorbed sera for comparison with absorption tests (Table IV) emphasized some of the differences pointed out above, so that it is probable that the agglutinative picture is not so confused as Table III would indicate. It is possible that by shortening the period of immunization of animals, as suggested by Tulloch (22), and using a less sensitive antigen, effective differences in agglutinations might be elicited even with this group of closely related strains.

Absorption Tests.—The organisms for absorption tests were grown in Kolle flasks on chocolate-blood meat infusion agar. Defibrinated human or cat blood was used as a common method of avoiding the blood used in the medium upon which the influenza bacilli had been cultivated for immunizing the animals, and the blood of the animals immunized, which were, of course, always identical. The flasks were seeded with 1 to 2 cc. of a young culture in hemo-peptone water. These cultures for seeding were transplanted daily as the impression was that such agglutinable cultures gave a growth which absorbed better than that obtained from seedings with old cultures transplanted weekly on blood agar. After the flasks had been incubated for 18 hours the growth was scraped off with a heavy nichrome spud and suspended in a minimal amount of salt solution. The resulting heavy emulsion contained at times small pieces of agar which were removed by filtration through a quadruple layer of cheese-cloth. Sedimentation was effected in the centrifuge for 30 minutes at 2,500 revolutions per minute in graduated tubes, after which the supernatant fluid was decanted and the volume of packed bacteria noted.

A few absorptions were made with the sera diluted to 1:50, but it was found that large doses of the organisms did not consistently remove the agglutinins for the absorbing strain. Then a 1:20 dilution of the sera was used with better results. To the packed bacterial

mass was added enough 1:20 serum to give two, three, or four parts of cells to one of undiluted serum, according to the titer of the serum used. This method of adding serum appeared more accurate than pipetting into each tube of material to be tested the small quantity of undiluted serum and then adding the requisite amount of salt solution. Of

TABLE IV.

Results of Agglutination Tests with Serum 6 Absorbed with Strain 8.

0.6 cc. of packed organisms to 0.4 cc. of serum; absorption in water bath for 3 hours at 55°C.; agglutination after absorption in water bath at 55°C. for 6 hours.

Strain No.	Salt solution control.	Dilutions of unabsorbed serum control.							
		1:100	1:200	1:400	1:800	1:1,200	1:1,600	1:2,000	1:2,400
1	—	+	+	+	+	+	+	+	+
2	—	+	+	+	+	+	+	+	+
6	—	+	+	+	+	+	+	+	+
8	—	+	+	+	+	+	+	+	—
9	—	+	+	+	+	+	—	—	—
11	—	+	+	+	+	+	+	+	+
14	—	+	+	+	+	—	—	—	—
15	—	+	+	+	+	+	+	+	+

Strain No.	Salt solution control.	Dilutions of absorbed serum.							
		1:100	1:200	1:400	1:800	1:1,200	1:1,600	1:2,000	
1	—	+	+	+	+	+	+	—	
2	—	+	+	+	+	+	+	—	
6	—	+	+	+	+	+	+	—	
8	—	—	—	—	—	—	—	—	
9	—	—	—	—	—	—	—	—	
11	—	+	+	+	+	+	—	—	
14	—	—	—	—	—	—	—	—	
15	—	+	+	+	—	—	—	—	

+ indicates agglutination; — no agglutination.

Strain 8 removes from Serum 6 the agglutinins for Nos. 8, 9, and 14.

course the final dilution would be slightly more than 1:20 and should be taken into consideration when the agglutinations are set up after absorption. Attempts to obtain the limits of specificity by using large doses of bacteria—seven or eight parts of bacteria to one part of serum—never gave a complete removal of heterologous agglutinins.

The elimination of the possibility of non-specific removal of agglutinins seemed certain as the proportion of bacteria to serum was never more than 4:1 and most of the time 2:1 or 3:1. Absorptions were made at 55°C. in the water bath for 3 hours, the tubes being shaken occasionally to insure good contact. Then the tubes were centrifuged for 30 minutes. The clear supernatant fluid was collected and stored on ice till the following day when agglutinations were set up, with the technique described above. The usual salt and serum controls were employed; the latter were made from serum which had been incubated, centrifuged, and let stand exactly as had the test material. The titer of a serum for the homologous strain was often reduced markedly, sometimes to one-fourth the control titer, when it was absorbed by a heterologous strain. These results were interpreted as negative in view of the complete removal of the agglutinins by a strain of the homologous group in similar dosage. The phenomenon occurred particularly with the meningitic strains which fell into different groups. This technique has been described at length because it is felt that an attempt has been made to standardize serological work upon influenza bacilli which may bear an important relation to the results obtained.

When Serum 6 was absorbed by Strain 8 it was found, as shown in Table IV, that the agglutinins for Nos. 8, 9, and 14 were removed, while those for the homologous strain and Nos. 1, 2, 11, and 15 were left. A similar result was obtained when Serum 6 was absorbed by Strain 9. These tests suggested at once a grouping of certain strains and it will be noted that the agglutinations in Table III may be interpreted to give support to this grouping with the exception of the behavior of Strain 14 toward Serum 9. A later agglutination test controlling absorptions from Serum 9 showed No. 14 to be agglutinated almost to the titer of this serum.

To prove a grouping each serum was absorbed with the strains which it agglutinated. The results are shown in Table V.

From Table V the following facts are apparent. (1) Strains 1, 2, 6, 7, 11, 12, and 16 are alike in removing from Sera 1, 6, and 11 agglutinins for the homologous strain. (2) Strains 8, 9, and 14 form a similar group. (3) Strain 15 is probably intermediate between these two groups and more closely allied to the second group than the other.

Strain 15 completely removes the agglutinins from no serum except its own for the homologous organisms, while on the other hand all of the strains of Group 2 and Nos. 2 and 11 of the other group remove completely the specific agglutinins from Serum 15. (4) Strain 17 falls in neither group nor with Strain 3. (5) When Strain 10 manifested agglutinative relations with either of the main groups these were not

TABLE V.

Results of Absorption Tests.

Groups.	Strain No.	Serum No. 1	6	11	15	8	9	14	10	3	4	5	13
1	1		+	+	−	−	−	−					
	2	+	+	+	+	−		−					
	6	+		+		−	−	−					
	7	+	+	+	−	−	−	−					
	11	+	+		+	−	−	−					
	12	+	+		−	−	−						
	16	+	+	+	−	−	−	−					−
Intermediate.	15	−	−	−		−	−	−	−				−
2	8	−	−	−	+		+	+	−				
	9	−	−	−	+	+		+					
	14				+	+	+						
	10		−										
	17		−			−				−			
	3												
	4												
	5		−		−								
	13				−								

+ indicates the complete removal of specific agglutinins from a serum by a strain; − indicates the opposite. Where a symbol is lacking no absorption was made. Nos. 1, 2, 6, 7, 11, 12, and 16 constitute Group 1; Nos. 8, 9, and 14 Group 2; No. 15 is intermediate.

confirmed by absorption tests. (6) Certain ambiguous agglutinative reactions of the four non-meningitic strains can be interpreted as group reactions.

DISCUSSION.

Valentine and Cooper (23) found two groups of two strains each among 171 respiratory strains. One of these groups was enlarged by Povitzky and Denny (18) to include five strains by the study of 90 additional ones. On the other hand, the latter authors found four of seven meningitic strains to be identical. There is nothing to indicate that the significance of this difference in grouping between the meningitic and the respiratory strains was appreciated in relation to the disease influenzal meningitis. In fact it appears that they look upon it as a matter of chance, as they say: "In cases of influenzal meningitis, we were so fortunate as to have discovered a dominant type of *Bacillus influenzæ*."

The grouping which we obtained is not in accord with that shown by Povitzky and Denny (18) with seven of these strains. They found that meningitic strains corresponding to our Nos. 7, 8, 9, and 16 were agglutinated by an antiserum to No. 9 and absorbed the agglutinins; that an antiserum to No. 10 agglutinated only the homologous organism; and that Nos. 11 and 12 were agglutinated by neither of the above sera nor did they absorb the specific agglutinins from Serum 9. We found, using many more sera than they, that of these seven strains, Nos. 7, 11, 12, and 16 were in one group, Nos. 8 and 9 in another group, and No. 10 stood alone. No explanation is offered for this difference in results.

The essential point, however, is that we have been able to confirm and extend their results, showing the existence of definite groups embracing the majority of the available strains of influenza bacilli isolated from cases of meningitis in which they occurred. This is especially marked in distinction to the apparent heterogeneity of respiratory strains as well as of the four blood culture strains which we have studied from children with pneumonia.

It is difficult without further work on more meningitic strains and without further careful comparison of these with many respiratory strains to state just what the results so far indicate. At any rate several possibilities are suggested. The most interesting one is that out of the large heterogeneous group of influenza bacilli a smaller group of organisms possessing similar cultural characteristics and

similar or closely allied serological reactions has become specialized parasites, which enables them to attack and produce in children a definite disease picture, influenzal meningitis. This is analogous to the meningitis produced by the meningococci which constitute a definite group among the Gram-negative diplococci. If the above possibility proves untrue the next most likely one is that certain groups of influenza bacilli more or less similar culturally and serologically, having risen to various levels of pathogenicity, can cause serious diseases in man, for example, septicemias, pneumonias, and meningitis, in this respect being similar to pneumococci and streptococci.

Out of the thirteen meningitic strains the results with only two (Nos. 10 and 17) interfere with the first possibility mentioned. Immediately the question is raised as to which will be called meningitic strains. The history of the patients from whom Nos. 10 and 17 were isolated is not available at this time. These strains were isolated, it is true, from patients with meningitis, but this may have been secondary to a mastoid infection or pneumonia. In that event the meningitis might be considered a complication of some other disease caused by a respiratory strain of *Bacillus influenzæ*. Nor is it known whether these two strains were obtained in pure culture. It may be that they attacked the meninges aided by pneumococci or streptococci rather than by powers of their own pathogenicity. At least one child seen at the Harriet Lane Home had a meningitis following a mastoid infection. Cultures from the mastoid and the spinal fluid revealed a hemolytic streptococcus and an influenza bacillus. That strain was lost before this work was undertaken. It will require further study of patients and of autopsy material in connection with the study of the cultural and the serological reactions of the influenza bacilli isolated from them before it can be said definitely that there is a group of these organisms which has a predilection for the meninges, producing a meningitis as meningococci do and not secondarily to mastoiditis, sinusitis, or pneumonia.

If influenzal meningitis proves to be analogous to epidemic meningitis a further effort should be made to produce a curative serum in an attempt to lower a mortality of 90 to 95 per cent.

CONCLUSIONS.

1. An attempt has been made to standardize the cultural and the serological work on influenza bacilli. As technique and knowledge improve cultural and serological characteristics may be shown to be different from those reported here. The results are constant enough for comparison as they were obtained under as nearly similar conditions as possible.

2. Of thirteen meningitic strains of *Bacillus influenzæ* isolated by different workers during a period of 7 years, eleven are alike culturally and fall into two groups by absorption of agglutinin tests; seven are in Group 1, three in Group 2, with one intermediate strain. Two strains stand alone culturally and serologically.

3. Four blood culture strains from children with pneumonia differ from each other culturally and serologically. When these strains show a relation culturally to members of the meningitic group this is not confirmed by serological reactions. When more respiratory strains are studied there may be found among them some similar culturally and serologically to the meningitic group.

4. Evidence has been set forth in favor of the possibility that a certain group of influenza bacilli may have risen to a level of pathogenicity to produce a disease picture known as influenzal meningitis. If this be true an attempt should be made to obtain a potent serum for treatment.

BIBLIOGRAPHY.

1. Coca, A. F., and Kelley, M. F., *J. Immunol.*, 1921, vi, 87.
2. Slawyk, *Z. Hyg. u. Infectionskrankh.*, 1899, xxxii, 443.
3. Cohen, *Ann. Inst. Pasteur*, 1909, xxiii, 273.
4. Wollstein, M., *Am. J. Dis. Child.*, 1911, i, 42.
5. Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.
6. Davis, D. J., *Am. J. Dis. Child.*, 1911, i, 249.
7. Anderson, R. A., and Schultz, O. T., *J. Exp. Med.*, 1921, xxxiii, 653.
8. Wadsworth and Wheeler, cited in Park, W. H., and Williams, A. W., Pathogenic microörganisms, New York and Philadelphia, 7th edition, 1920, 457.
9. Jordan, E. O., *J. Am. Med. Assn.*, 1919, lxxii, 1542.
10. Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665.
11. Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 50.
12. Rivers, T. M., and Leuschner, E. L., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 130.

13. Fleming, A., *Lancet*, 1919, i, 138.
14. Fildes, P., *Brit. J. Exp. Path.*, 1921, ii, 16.
15. Thjötta, Th., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 197.
16. Rivers, T. M., and Poole, A. K., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 202.
17. Rivers, T. M., *J. Am. Med. Assn.*, 1921, lxxvi, 1744.
18. Povitzky, O. R., and Denny, H. T., *J. Immunol.*, 1921, vi, 65.
19. Levinthal, W., *Z. Hyg.*, 1918, lxxxvi, 1.
20. Messerschmidt, Th., Hundeshagen, K., and Scheer, K., *Z. Hyg. u. Infectionskrankh.*, 1919, lxxxviii, 552.
21. Dreyer, G., *J. Path. and Bact.*, 1909, xiii, 331. Dreyer, G., Walker, E. W. A., and Gibson, A. G., *Lancet*, 1915, i, 324.
22. Tulloch, W. J., *J. Hyg.*, 1918, xvii, 316.
23. Valentine, E., and Cooper, G. M., *J. Immunol.*, 1919, iv, 359.

STUDIES ON THE TUBERCULIN REACTION AND ON SPECIFIC HYPERSENSITIVENESS IN BACTERIAL INFECTION.

By HANS ZINSSER, M.D.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

Plates 40 to 43.

(Received for publication, June 7, 1921.)

For a number of years we have been interested in defining, if possible, the rôle which anaphylactic sensitization to bacterial cell constituents might play in the symptomatology and pathology of infectious diseases. We have studied this problem from a number of different points of view and some years ago published, with Parker (1), a series of observations on the sensitization of guinea pigs with typhoid bacilli in which we made use of the isolated uterus method of Dale.

It has been quite apparent to all workers in bacterial anaphylaxis that the sensitization of animals to the cell materials of bacteria is much more difficult than is analogous sensitization with sera, egg albumin, etc., and it has usually been assumed, and we believe correctly, that this was due to the relatively small amount of coagulable protein in the bacterial cell.

In thinking about the problems of bacterial hypersensitiveness since that time, it has seemed to us of fundamental importance to obtain, if possible, a clear understanding of skin reactions such as the tuberculin, typhoidin, mallein, etc., tests, which represent more or less specific forms of hypersensitiveness in infected animals and man but, at the same time, differ in a number of important and perhaps fundamental aspects from phenomena generally associated with true anaphylaxis.[1]

[1] By true anaphylaxis we mean the reaction of protein hypersensitiveness, in which it is now generally agreed that an antigen-antibody union is involved in which the predominant rôle is played by the sessile antibodies, and which can be

I.

The Two Different Types of Skin Reaction.

A basic fact in regard to skin reactiveness is the occurrence, in man and in guinea pigs, of two fundamentally different types of such reactions, both of them to a considerable degree specific, yet differing both in the nature of the completed reactions and in the time factors concerned in their development. We specify guinea pigs and man because, in rabbits, the distinction is not so clear, a matter to which further reference will be made below. The two forms to which we refer are as follows:

1. The intradermal reaction, which appears in from 2 to 3 to 15 minutes after injection of the antigen. It expresses itself in the development of a growing wheal, often surrounded by a red areola of variable size. This reaction may last from $\frac{1}{2}$ hour to 1 or 2 hours, and fades again, usually without leaving any profound injury of the tissues. This is the reaction obtained with such substances as horse serum and has been extensively used in man to determine whether or not the particular individual was sensitive to horse serum when therapeutic injections were intended.

2. The other type of skin reaction is one in which there is no immediate effect, but in which within 4, 5, or more hours, a swelling becomes apparent which in the course of 12 to 24 hours results in a swollen, edematous area of varying intensity, often with a central necrotic spot and, occasionally, hemorrhage. This reaction may not reach its highest development until about 48 hours after the injection, and is accompanied by distinct signs of inflammation and some cell death.

passively and specifically transferred to normal animals with the serum of other animals as long as this serum contains antibodies. We do not wish to take up space with this definition, but refer the reader to the recent and excellent summaries of Doerr (Doerr, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 947), Coca (Coca, A. F., in Tice, F., Practice of medicine, New York, 1920, i, 107), and Wells (Wells, H. G., *Physiol. Rev.*, 1921, i, 44). Although differing somewhat in nomenclature and in classification, the three summaries lay down fundamental principles which are essentially alike and with which we agree.

The classical examples of such skin reactions are the tuberculin intradermal test, the typhoidin reaction, the mallein reaction, and the reactions studied by Fleischner, Meyer, and Shaw (2) with *Bacillus abortus* and *Bacillus melitensis* in guinea pigs.

As we shall see, the two reactions may occur in the same animal, after one and the same injection, but, in guinea pigs, can usually be differentiated, since one begins to fade before the other appears.

It seemed that our first task, then, was to determine whether either one of these varieties of skin reactions or perhaps both of them could be correlated with true anaphylaxis.

Relationship of the "Immediate" Skin Reaction to Anaphylaxis.

The immediate skin reaction has been used for some years as an index of safety before the injection of therapeutic horse serum in the specific treatment of pneumonia and other diseases. Its application in this connection was based primarily upon the publications of Moss (3), of Knox, Moss, and Brown (4), of Longcope and Rackemann (5), of Mackenzie and Leake (6), and of others. It should be noted, however, that the work of Moss and his collaborators, on which the assumption that the skin reaction was a reliable index of general hypersensitiveness is based, was done with rabbits, and in rabbits the skin manifestations of anaphylaxis, both intracutaneous and subcutaneous (Arthus phenomenon) are always in the form of delayed reactions, for reasons that we believe depend upon peculiarities of the rabbit, rather than upon fundamental differences in the nature of the reaction. This peculiarity in rabbits, however, is being investigated in our laboratory at the present time in a study on the Arthus phenomenon and will be reported upon subsequently. Meanwhile, it seemed to us necessary, since we were working with guinea pigs, to determine whether in these animals an immediate skin reaction to a protein antigen such as horse serum, egg albumin, etc., could be elicited at all, and, if so, whether its development was parallel to that of general protein hypersensitiveness.

In consequence, we carried out a number of experiments in this direction.[2]

[2] These experiments were done with the assistance of Dr. S. T. Woo and Mrs. J. T. Parker, who was carrying out skin reactions in guinea pigs for another purpose at the time.

Series of guinea pigs were given from 1 to 2 cc. of horse serum intraperitoneally. At the end of from 2 to 3 weeks, they were given intracutaneous injections of horse serum in dilutions ranging from 1 : 5 to 1 : 20. Normal controls were always injected at the same time and intravenous injections of varying quantities of horse serum were made on guinea pigs similarly sensitized in order to determine general anaphylaxis. We omit protocols since the experiments were extremely simple and we do not wish needlessly to prolong this paper.

Our results may be stated briefly as follows: Guinea pigs in the sensitized condition, that is 2 to 3 weeks after the intraperitoneal injection of 1 cc. of horse serum, will generally, though not always, show an immediate reaction to the intracutaneous injection of 0.1 cc. of a 1:10 or 1:20 dilution of horse serum.

There is a considerable increase in the size of the wheal formed at the point of injection, the increase beginning usually within 5 or 10 minutes, and increasing up to from ½ to 2 hours, then gradually fading. It was usually completely gone in from 2 to 4 hours and in most cases was accompanied by a red areola quite comparable to the reaction observed in human beings under similar conditions. These reactions never caused any serious or lasting injury to the tissues.

This reaction was roughly parallel to the sensitiveness of the guinea pigs to intravenous horse serum injections.

It can be produced in guinea pigs by passive sensitization, appearing on the 2nd day after the injection of the anti-horse rabbit serum almost as well as in the actively sensitized guinea pigs.

Guinea pigs that have been shocked and become antianaphylactic are desensitized to skin reactions made on the following day.

Relationship of the Delayed Tuberculin Type of Skin Reaction to Anaphylaxis.

Having thus determined that immediate skin reactions to horse serum in guinea pigs might be regarded, with reasonable certainty, as one of the manifestations of general hypersensitiveness, we next proceeded to investigate the same point in connection with the second type of reaction; namely, that represented by tuberculin, mallein reactions, etc. We selected tuberculosis and the tuberculin reaction as the first type to be studied because with tubercle bacilli it is relatively easy to produce, at will, infections of varying intensities and different speeds of progression, and since the volume of clinical

data and the careful studies of tuberculin skin reactions in guinea pigs by Baldwin, Krause, and others had cleared the way for a free approach to the basic problems. The reaction, moreover, is essentially the same type of occurrence as that following the injection of typhoidin, mallein, or abortin preparations in animals respectively infected with the organisms from which the antigens have been produced.

It is quite well known that in an interpretation of reactions such as the tuberculin reaction, the field is fairly divided between those that hold these phenomena to be manifestations of true anaphylaxis and those who deny this.

Calmette (7), who separates the tuberculin reactions distinctly from anaphylaxis, nevertheless states that tuberculous animals or man develop a lytic principle, presumably in the nature of a bacteriolysin, which goes into reaction with the specific antigen in the tuberculin preparations. This fundamentally is, in a sense, an anaphylactic conception. Friedberger has frankly regarded the phenomenon as an anaphylactic one, developing his ideas along his theories of anaphylatoxin formation.

Study of the reactions from a theoretical point of view was begun by Römer (8) in 1909, and by Baldwin (9) in 1910. Baldwin's work is fundamental, in showing that, in spite of previous assertions, guinea pigs could not be rendered skin-sensitive by implantation of porous filter capsules or celloidin capsules containing tuberculoprotein, or living tubercle bacilli. He showed that skin sensitiveness could never be produced without actual infection with living organisms. Animals treated with tuberculoprotein, however, often showed reactions to intravenous inoculation of the homologous preparation which could be recognized as anaphylactic. His conclusions can be summarized as follows:

Tuberculous animals become sensitive to anaphylactic test, but not uniformly so. There is no absolute relation between the degree of sensitiveness and the stage of the disease. Injections of the tuberculoprotein may sensitize normal guinea pigs. Sensitized guinea pigs, however, do not react to the ordinary tuberculin test, though some respond slightly to the intradermal test. He adds: "This difference between anaphylactic sensitization and tuberculin reactivity need not be fundamental, as it is possibly due to another factor as yet undetermined." His experiments on the transfer of passive anaphylaxis to tuberculoprotein were inconclusive, but it has been shown since then, by Austrian (10) and others, that passive sensitization can be attained. From a theoretical point of view the most important observation of Baldwin is the fact that there seemed to be a discrepancy between skin sensitiveness and general anaphylaxis. Krause (11), following out the work of Baldwin, confirmed and extended the above observations and established an interesting relationship between skin sensitiveness and the progress

of infection. He asserts that skin sensitiveness develops simultaneously with the development of the initial focus, increases progressively with the lesions, varies directly with the extent and intensity of the infection, and diminishes with healing. It is blunted by a general tuberculin reaction which suggests analogy to saturation, such as that which occurs in connection with anaphylaxis.

In dealing theoretically with the tuberculin reaction, especially as regards the possibility of its being an anaphylactic phenomenon, it is of great importance to determine whether or not the reaction can be transmitted passively. The evidence on this is contradictory. Only a few writers have reported positively in this connection. In 1909, Bail (12) injected finely divided tissue mash of tuberculous organs of guinea pigs into normal guinea pigs, and 24 hours later gave the animals so treated 0.5 cc. of old tuberculin, or a preparation which in this quantity had practically no effect upon normal animals. The animals prepared with the tuberculous tissue died in some cases, while controls treated with normal tissue suspensions showed no symptoms.

Helmholz (13) in the same year reported positive skin reactions in normal guinea pigs 2 to 6 days after he had injected them intraperitoneally with the defibrinated blood of tuberculous guinea pigs. Both of these observations would be of fundamental importance if they could be confirmed.

In considering the mechanism of the tuberculin reaction, it will be well to examine also the work that has been done on the typhoidin reaction. Gay and Claypole (14) believed that positive skin reactions in rabbits were parallel with the degree of immunity of the animal. They succeeded in transferring the susceptibility to typhoidin from an immune to a normal animal by inoculation of 20 cc. of typhoid-immune serum, testing 24 hours later. These experiments were repeated and confirmed by Meyer and Christiansen (15); and in their first work with rabbits, these last observers, using what they called a typhoid autolysate (by which they mean an alcohol precipitate of a heated distilled water suspension of a 48 hour agar culture), concluded that "the typhoidin and similar reactions in rabbits are anaphylactic in nature and the result of an interaction of antigen and antibody." They stated that "the logical assumption from these facts is than cutaneous hypersensitiveness is the result of bacterial protein sensitization." Later Meyer (16) found that injected rabbits react with typhoidin more intensively than do immunized rabbits, and drew the conclusion that cutaneous hypersensitiveness does not indicate that the rabbit is particularly immune, and that no definite relationship existed between agglutinins and complement-fixing antibodies and skin sensitiveness. From these first two papers of Meyer's we gather that he believed that in rabbits skin sensitiveness to typhoidin is a sign of infection, rather than of immunity, but that as stated in his own words "cutaneous hypersensitiveness of rabbits . . . . . is, in all probability, the result of sensitization with typho- or similar bacterial proteins." Nichols (17) also considered the typhoidin reaction as he observed it in human beings as a protein sensitization.

The apparent discrepancies between the results of Baldwin with the tuberculin reaction and those of the workers just mentioned with the analogous typhoidin reaction, probably depend upon the fact that Baldwin used guinea pigs and the other observers used rabbits. When, subsequently, Fleischner, Meyer, and Shaw (2) studied cutaneous hypersensitiveness in guinea pigs treated with repeated intraperitoneal injections of *B. abortus*, *B. typhosus*, and old tuberculin, and carried out parallel experiments with animals infected with living organisms, the conclusions that they reached coincided with those of Baldwin in the case of the tuberculin reaction. They found, in other words, that guinea pigs treated with dead bacterial proteins might become anaphylactic, but did not give skin reactions.

It will be seen from this brief review of the literature that, in spite of a great deal of careful work by experienced observers, there is still a considerable degree of confusion. We considered it best, therefore, in studying the tuberculin reaction to begin at the bottom and reexamine the conditions in all their details, substituting reactions with the isolated guinea pig uterus for the intravenous test for anaphylaxis. This point is particularly important since the intravenous injection of bacterial extracts into guinea pigs is apt to give rise to confusing symptoms; and in carrying out such experiments we have again and again felt uncertain as to whether or not an individual, mild reaction should be interpreted as a feeble manifestation of anaphylaxis, as due to toxicity of the bacterial preparation, or perhaps even as the result of the injection of finely divided colloidal particles.

The first stages of our work, then, concern themselves with a study of tuberculous guinea pigs and guinea pigs treated with various tubercle bacillus extracts, determining in both cases the relationship of intracutaneous tuberculin reactions with anaphylaxis as manifested by tests with the isolated uterus. We summarize the final procedures in order to avoid a needless account of much preliminary groping for a suitable technique.

Technique.—The tubercle bacillus used throughout was a human type of moderate virulence originally isolated, we believe, at Saranac. Many different methods of extraction were used, but the preparation with which most of the work was done was made as follows:

About 100 mg. of ground and powdered tubercle bacilli were shaken up in a shaking machine for 3 to 4 hours, with 200 cc. of salt solution to which had been added enough normal sodium hydroxide to give a final concentration of 0.2 per cent of the alkali. When the powder was added to this, the buffers reduced the alkalinity, which finally ranged somewhere about pH 8. After shaking, the bottles

were preserved in the ice chest, and sometimes reshaken on subsequent days. The gross particles were removed before this was used, either by filtration through a Berkefeld filter, or by high speed centrifugation, and the slightly opalescent fluid was the basic preparation finally employed in most of the experiments, and fractionated subsequently for special purposes. In sensitizing guinea pigs it was very often the unfiltered extract which was intraperitoneally injected without centrifugation.

The following method was found to be the most suitable for the sensitization of normal guinea pigs with the tubercle bacillus extracts. Young female guinea pigs of about 150 to 200 gm. weight received from 1 to 2 cc. intraperitoneally, at first on alternate but in later experiments on successive days, until ten to twelve injections had been given. It was found best to test these guinea pigs not earlier than 20 days and usually not later than 28 days after the last injection.

Skin reactions were invariably done intracutaneously with a No. 26 gauge needle on a tuberculin syringe, and it was attempted to inject about 0.05 cc., but it was found that the concentration rather than the actual amount was the thing that seemed to count most. We gauged the skin reactions largely on the size of the wheal produced, attempting to produce the same sized wheal in comparative tests. It is next to impossible in guinea pigs to inject absolutely accurate quantities in separate tests, but, as a matter of fact, the variations in quantity injected could never have been very great or significant.

The uterine tests were all done in the same way, in a bath of 200 cc. of Ringer's solution with 0.5 to 1 per cent glucose and oxygen bubbling through the bath.

Studies with Tuberculous Guinea Pigs.

Guinea pigs were intraperitoneally injected with relatively large doses of tubercle bacilli, without any attempt to equalize the doses or to grade them, since we wanted to perform the test on guinea pigs with varying degrees of tuberculous involvement and to check up by autopsy after the results had been obtained.

Skin reactions were done on these animals with the undiluted but filtered extracts described above, and with old tuberculin in dilutions usually of 1 : 5 or 1 : 10, at varying intervals from the 1st day after infection. When definite skin reactions appeared, some of the guinea pigs were killed and the uteri put into the Dale apparatus, the extract mentioned above being used in most of these experiments, since, in working with old tuberculin, the margin between the nonspecific action on the normal uterus and the specific action on the sensitized uterus is a relatively narrow one, a thing also found by Weil (18), who is, as far as we know, the only one who has applied this method to tuberculosis, his report dealing with some isolated experiments carried out in 1917.[3] A great many animals

[3] Weil obtained uterine reactions with old tuberculin 9 weeks after injection with 0.001 cc. of old tuberculin, while the normal uterus did not react to less than 0.3 cc. The capacity of the bath is not given. He states that the reaction is an irregular one and occurs "at some stage of the disease."

were tested in this way, and the results were perfectly consistent, so that it is unnecessary to go into detailed study of the protocols. Whenever uterine tests were done, the tubercle bacillus extract used was titrated on the same day against normal uteri to make sure that we did not approach the non-specific limit, which varied considerably in these extracts. Also, no skin reactions were ever carried out without being accompanied by tests, with the same material in the same quantities, on normal guinea pigs, since, in working with alcohol precipitates and other chemically manipulated substances, mild non-specific reactions will occasionally appear.

The question to be answered was the following. In the course of a tuberculous infection does true anaphylaxis to tubercle bacillus products develop, and, if so, is it parallel in time and in intensity with the intradermal tuberculin reaction?

Figs. 1 to 3 illustrate the results obtained. Positive skin reactions were usually obtained within 9 or 10 days after inoculation, the earliest, observed by us, being between the 6th and 7th days. At this time the animals were never anaphylactic. Fig. 1 shows complete lack of anaphylactic reaction in the uterus in an animal 10 days after inoculation, the day after a positive skin test had been obtained. Fig. 2 shows two uteri simultaneously put into the bath, both of them 9 days after inoculation with tubercle bacilli, one giving an entirely negative skin reaction, the lower one giving a very marked ++++ reaction. Neither showed a sensitive uterus.

Fig. 3 shows a different state of affairs; namely, an animal inoculated subcutaneously with tubercle bacilli and tested 3 weeks after inoculation. This animal showed a powerful anaphylactic, as well as skin reaction. These records and a considerable number of similar ones indicate: (1) that guinea pigs suffering from infection with tubercle bacilli may become both skin-reactive and anaphylactic; (2) that the skin reaction develops early and may exist without any detectable signs of general anaphylaxis as evidenced by the uterine reaction; (3) that within 3 weeks or later, after the animals are still in fairly good condition, the skin reaction and the uterine reaction may coexist.

It is during this period that a simulated parallelism may have led others, working with a less delicate anaphylactic method, into confusion. Towards the end when the guinea pigs are moribund, both the skin reaction and general anaphylaxis may fade, another feature

which simulates parallelism; but many reactions like those above have convinced us that there is no question about the correctness of Baldwin's original contention, that the two conditions, skin reactiveness (and probably, therefore, the other forms of tuberculin hypersensitiveness) and anaphylaxis do not necessarily coincide.

Studies on Guinea Pigs Sensitized with Materials from Killed Bacteria.

The next question to be answered was that concerning the conditions existing when guinea pigs were not infected, but when they were treated with products of dead tubercle bacilli. Young female guinea pigs were prepared by the method of sensitization outlined above. They were run through in sets of six or more. Between July, 1920, and January, 1921, five sets of such guinea pigs were run through, all of them treated with ten or more injections of extract. Skin reactions were done on these animals, both during the process of sensitization, that is in the course of the 10 to 20 days during which they were being treated, and at varying times thereafter, up to the period of 3 weeks after the last injection when they were tested for uterine sensitiveness.

Practically none of these guinea pigs showed typical delayed skin reactions comparable to the true tuberculin reaction. In only two cases did we see reactions which might have been regarded as moderate tuberculin reactions. It may be noted that Baldwin also observed a few exceptions to his other negative results. An example of one set in which these exceptions occurred may be worth recording since it will typify our general procedure.

Experiments on the Sensitization of Guinea Pigs with Tuberculoprotein.—Six guinea pigs (Nos. 1 to 6), were injected intraperitoneally with 1 cc. of unfiltered tubercle bacillus extract as follows: September 1, 3, 7, 9, 11, 13, 20, 28, 1920. The first skin reactions were done on four of the guinea pigs with two preparations of old tuberculin diluted 1 : 5, and undiluted extract similar to that injected on September 28. Negative results.

October 2. All the guinea pigs were reinjected with 2 cc. of the extract; skin reactions on this day were negative.

October 7. Skin reactions done on three of the guinea pigs at the same time with four normal controls and one tuberculous control. When these reactions

were read on October 8, Guinea Pig 2 showed what ordinarily we should have called a +++ reaction. It should be mentioned that this guinea pig was very much emaciated and we were suspicious of its having been spontaneously infected with tuberculosis. It was killed and autopsied, and a few small yellowish spots on the surface of the liver about 2 mm. in size at the base were taken for section and examined,[4] but could not be identified as tuberculous (we may mention that this occasional appearance of small knob-like lesions on the liver of extract-treated guinea pigs has not been uncommon). The uterus of this animal was found to be moderately sensitive. Subsequent tests of the rest of these guinea pigs showed only one other suspicious skin reaction, less marked than that on No. 2.

The isolated uteri of these extract-treated guinea pigs were, with very few exceptions, found to be sensitive to extract anywhere from 20 to 30 days after the last injection. Fig. 4 shows two guinea pigs in which positive, typical reactions were obtained with the uteri with 4 cc. of a rather weak extract which in these quantities had no effect upon normal uteri. In both of these animals, the skin reactions had been consistently negative.

It may be noted in passing, however, that although these extract-treated guinea pigs did not show the typical tuberculin reaction with well defined swollen areolæ, etc., after 24 hours, they did occasionally show an immediate reaction not incomparable to the immediate reaction described above for horse serum. Whenever such reactions were observed, they occurred at about the time when the guinea pigs showed anaphylactic reaction to uterine test. This is mentioned in passing as showing that even with the products of the tubercle bacillus the two kinds of skin reaction can be elicited.

Moreover, the two graphic records shown in Fig. 4 are only two of very many in which typical uterine reactions were elicited in guinea pigs similarly prepared with considerably smaller quantities of various kinds of tubercle bacillus preparations. It will be seen in the lower record that a repetition of the extract showed the uterus to be desensitized, a matter which has been noted many times.

Therefore, from a considerable number of experiments similar to those described in perfect accord with the results obtained by Baldwin by other methods, we may conclude that delayed reactions, of the tuberculin reaction type, may develop independently of generalized anaphylaxis in the ordinary sense in which this word is used, may be

[4] The examination was made by Dr. Frederick Parker.

present in tuberculous guinea pigs before anaphylaxis to tuberculoprotein has developed, and are with very few exceptions entirely lacking in guinea pigs rendered anaphylactic by sensitization with tubercle bacillus products.

These facts, then, definitely confirm the opinion, first clearly advanced by Baldwin, that tuberculin hypersensitiveness may develop independently of general tuberculoprotein anaphylaxis, and that the former type of hypersensitiveness is associated particularly, perhaps solely, with the existence of an infection. This, too, is in complete keeping with the experience of Fleischner, Meyer, and Shaw who found that intradermal tests were positive only in guinea pigs infected with the bacillus of bovine abortion, but consistently negative in these animals intensively immunized by intraperitoneal injections of dead organisms, or with extracts of organisms.

II.

The fundamental facts may then be stated as follows: Under the influence of contact with living tubercle bacilli, and probably other bacteria, two distinct varieties of hypersensitiveness develop in guinea pigs. One of them, true anaphylactic hypersensitiveness, develops late and can be readily induced by appropriate treatment with dead bacterial materials, extracts, etc. The other, the typical tuberculin reaction (and probably the mallein, typhoidin, and abortin reactions) may be regarded as infection phenomena, for, while we may eventually succeed in inducing these reactions by modified methods of sensitization,[5] they are most easily and characteristically elicited as soon as an infection is established, while, to date, they have but rarely and atypically followed upon any form of artificial sensitization. And yet, in spite of the apparent mutual independence of these two forms of hypersensitiveness, both reactions, or either one of them alone, may be elicited with one and the same bacterial extract in appropriately prepared animals.

This forces us to inquire whether a single constituent of such bacterial extracts is responsible for both types of reactions, or whether it is possible to separate, from such preparations, two functionally

[5] See below.

distinct fractions, one capable of eliciting the typical anaphylactic response, the other active in the tuberculin sense.

The latter opinion is rendered likely by the generally accepted view that the true anaphylactinogens are proteins, whereas most of the work done with tuberculin seems to indicate that these substances are of a simpler structure.

Löwenstein and Pick (19) among others conclude that the active substance in tuberculin is a protein-free, alcohol-precipitable substance which is not coagulated by boiling either in neutral or acid reaction, cannot be precipitated with ammonium sulfate, gives a negative biuret reaction, but is precipitated with tannic acid. We will not go into the details of analysis, but their final conclusion is that the active substance is not a protein, but belongs in the class of protein split products which Fischer has spoken of as "polypeptides." They claim that dialysis of the concentrated tuberculin solution weakens it after a short time, and that after prolonged dialysis its activity is entirely lost; they do not, however, state the nature of the membranes used, nor the particular conditions under which dialysis was performed. The active tuberculin substance, moreover, which Calmette uses for his ophthalmoreaction is the white, flocculent, and highly soluble substance which is precipitated out of tuberculin solutions upon the addition of ten or twenty volumes of absolute alcohol.

Along the lines of these investigations we proceeded to attempt separation of the various substances which might be obtained from tubercle bacillus extracts.

Attempts at Chemical Separation of the Tuberculin from the Anaphylactinogen.

From powdered tubercle bacilli of the human type[6] we produced extracts by shaking in a 0.02 per cent sodium hydroxide solution in physiological salt solution. 3 or 4 hours shaking and perhaps a day or so in the ice chest sufficed to bring a considerable amount of the material into solution. This extract was centrifugalized until all the particles had been removed and a moderately opalescent supernatant fluid was decanted.

This material, upon being acidified to approximately pH 5 to 6, with 2 per cent acetic acid in the cold, became turbid and soon precipitated in large flakes. Further acidification up to pH 4 did not redissolve these flakes. The precipitate represented the bulk of the dissolved substance in the extracts. The precipitate could be redissolved in a slightly alkaline salt solution and reprecipitated with

[6] These bacilli were kindly furnished by Dr. S. A. Petroff of Saranac.

acid. Because of their precipitability by acetic acid in the cold, we designated these substances as nucleoproteins or phosphoproteins, although we do not wish to commit ourselves, chemically, since we are aware of the indefiniteness of these biochemical terms and realize fully our incompetence to deal authoritatively with a chemical problem of such difficulty, without further intensive study.

After removal of these acid-precipitable substances by centrifugation and filtration through Berkefeld candles, the fluid was brought to a boil in the acid condition, and sometimes a very faint turbidity developed which was taken to represent the presence of coagulable protein, albumin or globulin, or both. These precipitates were so fine and slight that only Berkefeld filtration would remove them, and even this was not always completely successful.

The fluid was then neutralized; that is, brought to an approximate pH of 7.

When this neutral fluid was filtered hot, we observed on numerous occasions that a slight precipitate developed over night in the ice chest, and that this precipitate redissolved on heating, a point which indicated the possibility that the tubercle bacilli may contain Bence-Jones protein. This point, however, will need further chemical analysis, a task which we have not yet had time to undertake. The water-clear fluid which was left after removal of all these substances gave in all cases a very definite precipitate with alcohol. The precipitate could be thrown down, collected, and redissolved in water with salt solution, and like the similar precipitate obtained from crude tuberculin was astonishingly soluble.

This final water-clear material gave no biuret reaction, and usually gave no sulfosalicylic acid reaction, though occasionally this was very faint; in most cases it gave no Millon reaction, was not clouded on boiling with acid, and usually gave no xanthoprotein reaction, though in some cases a slightly yellowish color appeared when the ammonia was added in the second part of the test. This material, for want of a better name, we speak of as the "proteose" residue.

Having thus fractionated the original tubercle bacillus extract, we proceeded to carry out biological reactions with various parts, comparing chiefly the whole extracts with the dissolved substances which precipitated with acid in the cold (nucleoproteins) and with the final solutions from which the acid-precipitable, acid- and heat-coagulable substances and the material suspected of being Bence-Jones protein had been removed (proteose residue).

We have done a large number of skin reactions on tuberculous guinea pigs with the apparently protein-free proteose residue, always controlling with tests on normal guinea pigs. This precaution is absolutely necessary, since we have found (as we were warned to expect by Baldwin) that repeated alcohol precipitation or prolonged boiling in an excessively acid reaction (pH 4 or below) may eventually

lead to non-specific toxicity, both for the skin test and for the isolated uterus.

Invariably this final proteose material gave skin reactions, often quite as powerful, and never more than slightly less than the original whole material. And, indeed, it was to be expected that a certain amount of the tuberculin active substance might be carried down in the flocculent precipitate formed when the acid is first added, so that the solution is thus deprived of a certain percentage of its activity. The protocol presented in Table I is an example of experiments of this kind.

TABLE I.

Skin Reactions Read after 24 Hours.

No. of tuberculous guinea pigs.	Whole extract 1:2.	Proteose 1:2.	Whole extract 1:5.	Proteose 1:5.
7	++++	++++	+++	++
8	+++	++	+++	+++
9	+++	++	+++	++
	Whole extract undiluted.	Proteose undiluted.		
10	++++	+++		
11	+++	+++		

Two normal controls show no non-specific reactions.

++++ indicates a very marked reaction; +++ a large reaction, slightly less than the preceding; ++ a moderate reaction; + a definite but faint reaction; ± doubtful; − negative.

Reactions of this nature, often repeated, indicate definitely that in removing the acid-precipitable and the coagulable protein substances from the tubercle bacillus extracts, we did not remove any considerable part of the skin-reactive substance. Moreover, they justify the assumption that, in the tuberculin skin reactions, the antigen is not necessarily a protein substance, but probably belongs in the category of the so called proteoses or perhaps polypeptides.

It must be stated, however, that the nucleoprotein fraction, precipitated from the original extracts with acid in the cold, always retained tuberculin activity. In spite of repeated reprecipitation and resolution, we have never succeeded in entirely removing the capacity

of inducing skin reactions of the tuberculin type from these acid-precipitable substances. This may be due to the adsorption of the proteose material by the heavy flakes of the precipitate. On the other hand, subsequent experiments suggested the possibility that these nucleoproteins (?) which constitute the bulk of the soluble material of the bacterial cell might represent the mother substance from which the proteose materials are derived.

However this may be, the proteoses alone are fully capable of giving rise to the typical tuberculin reaction in the infected guinea pigs.

This being the case, what is the effect of this proteose fraction upon the isolated uterus of the extract-sensitized or anaphylactic guinea pig in which no tuberculin skin reaction can be elicited?

In these experiments our results were often not so clean-cut as they might have been. Nevertheless, in a considerable number of tests there was indication that the proteose fraction, powerfully skin-reactive, had little or no effect upon the uteri of extract-sensitized animals, which contracted powerfully when brought in contact with the whole extracts or the acid-precipitable fractions in amounts that had no effect upon the normal uterus.

Fig. 5 shows the uterus of a guinea pig, sensitized with extract, which was unaffected by 2 and 2.5 cc. of the proteose fraction, but contracted powerfully and went into a spasm when 1.5 cc. of the whole extract were added to the bath.

Fig. 6 shows a similar experiment in which 2.5 cc. of the proteose fraction had no effect, whereas as little as 1 cc. of the acid-precipitable fraction produced a strong contraction and spasm.

A number of records (six) as sharp and unambiguous as the above were obtained. But in addition, we must state that on three occasions the proteose material contracted the uteri of highly sensitized and of tuberculous guinea pigs when relatively large amounts of proteose were used. Whether this means that we had not freed the proteose completely of protein, or whether it signifies that, with sufficiently energetic treatment, the proteoses, too, may form antibodies, is a question that cannot be definitely answered. We incline to the latter view for reasons that will be discussed below.

Notes on the Dialysis of Tuberculin.

It will be remembered that the chief clinical difference between the immediate skin reaction of the anaphylactic type and the tuberculin type of reaction is the fact that the former consists in a rapidly developing urticaria-like swelling which disappears in a relatively short time, and leaves little or no injury behind. The latter, however, comes on slowly, and when once developed is accompanied by manifestations of inflammation and, eventually, profound injury of cells, often with cell death, a state of affairs which at least suggests the penetration of the injurious substances into the cells themselves. This, taken together with our knowledge of the relatively simpler structure of the tuberculin substances and Löwenstein and Pick's claims concerning their diffusibility, suggests a number of interesting lines of reasoning.

Is it not possible that the diffusibility of antigenic substances is a very important factor not only in the reaction of these diffusible substances with the cells, but also in the formation of antibodies? Is it not likely that substances which are practically non-diffusible should be excluded from direct reaction with the tissue cell and that the mechanism of antibody formation is a device for a reaction with non-diffusible materials? And, if this is so, is it reasonable to suppose that the reaction of the body to substances which are relatively more diffusible should become increasingly intracellular, as diffusibility increases, and that antibody formation, in the usual sense of the word, should, therefore, become less and less essential as relative diffusibility increases? Such a view would explain at the same time the apparent intracellular nature of such reactions as the tuberculin reaction and the difficulties encountered in attempts at passive transfer of such sensitiveness. Accordingly, we thought it worth while to attempt to separate by dialysis the substance which produced the tuberculin skin reaction from that which caused typical anaphylaxis.

In planning experiments upon the diffusion of antigenic substances through semipermeable membranes, we are quite aware of the many pitfalls which entrance into such a field on the part of the biologist implies. We know that it is by no means proven that the living cell membrane is at all permeable to substances like the proteoses and that experiments with dialysis *in vitro* may lack some of the essential criteria that govern dialysis in the animal body. Nevertheless, it seemed worth while to investigate the diffusibility of the tuberculin active substances. We at first tried to work with membranes graded by the methods of Brown (20) and of Gates (21). But since the differences in diffusibility of proteins and the proteose-like substances with which we are concerned can be very slight ones only, we abandoned this and worked empirically, attempting, in many tests, to find membranes which would let through the tuberculin active substances, but hold back the anaphylactinogenic ones.

The general procedure consisted in taking various tubercle bacillus preparations, the skin reactiveness of which had been determined on tuberculous guinea pigs, and subjecting them to dialysis either in celloidin or in fish bladder bags, in

a closed system, testing the material outside the bag from time to time on tuberculous and normal guinea pigs. As soon as the material outside gave indication of containing the skin-reactive substance, the same material was used upon a sensitized and a normal uterus. In a few experiments we obtained results which at least indicate that the tuberculin active substance is more readily diffusible than the anaphylactinogens.

Experiment 1.—A glycerol salt solution extract of powdered tubercle bacilli was started on February 7, 1921. 1 gm. of powdered tubercle bacilli was infused in 500 cc. of a 5 per cent glycerol salt solution, heated to 70°C. for 1 hour, and placed in the incubator over night. It was then shaken for several hours on consecutive days. This material was simmered down to 150 cc. at a temperature of 75 – 80°, never quite being allowed to come to 80°, and was then filtered through paper. It gave a strong sulfosalicylic acid reaction. February 15. 40 cc. of this material

TABLE II.

Readings on Skin Reactions Done February 17.

Guinea pig No.	Skin reactions.	
	With material outside bag (B_2).	With material inside bag (B_2).
12	++	++++
13	++	++++
14	++	++++
15	−	++++
16	±	++
17	±	++
18	+	+++
Normal control.	−	±
" "	−	±

were placed in a fish bladder bag in a closed system with 40 cc. of salt solution outside. February 17. Skin reactions were done with the material inside and outside the bag, and it was found that a very definite though mild skin reaction resulted from the material outside the bag. The relative strength of the skin reaction with material inside and outside the bag are given in Table II.

February 18. This material was tested upon normal uteri and it was found that 2.5 cc. of the original material and the material outside the bag, added to a 200 cc. Ringer's bath in which a normal uterus was rhythmically contracting, produced no marked changes in the rhythm. The material was then tested on the uterus of a guinea pig sensitized as above described with tubercle bacillus extract. As will be seen in Fig. 7, 4 and 6 cc. of the material outside the bag, which gave a skin reaction, produced no spasm of the sensitized uterus, whereas 2 cc. from inside the bag gave a typical and marked reaction. We realize that this may have been a quantitative result, due to a greater sensitiveness of the skin than of the uterus.

This does not seem likely under the circumstances, but, nevertheless, must be considered.

We have noted above that Löwenstein and Pick claimed that they could completely free old tuberculin of its activity by prolonged dialysis against running water. For this reason, we determined to try the reverse experiment; that is, attempting by prolonged dialysis to remove the skin-reactive substance entirely from the inside of a dialyzing bag, leaving the anaphylactinogenic substance behind. We tried this with various preparations, but never succeeded even with prolonged dialysis in freeing the materials on the inside of the bag entirely of a skin-reactive substance, although such a result had been claimed by Löwenstein and Pick. In one case only did we obtain a result worth reporting. We placed 25 cc. of concentrated old tuberculin from the Department of Health of the City of New York in a fish bladder dialyzing bag, and dialyzed this against running water. The old tuberculin used still gave a powerful sulfosalicylic acid reaction for protein, and gave a definite precipitate on being acidified with acetic acid in the cold after dilution to about 1 : 20. The precipitate did not redissolve upon heating, but was rather intensified. With this preparation we had what we must now consider a rather fortunate accident. Because we were dialyzing with running water, we could not carry out the procedure in the refrigerator, and bacteria began to grow in the dialyzing bag. In the course of 5 days, the material from the inside of the bag gave practically no skin reaction on tuberculous guinea pigs as compared with old tuberculin diluted 1 : 3, which corresponded to approximately the dilution which had been attained by the substance in the course of dialysis. This material which no longer gave skin reactions was tested against normal and sensitized uteri, and it was found that, although 3 cc. of the material gave no reaction on the normal uterus, powerful reactions with sensitized uteri were obtained in quantities as low as 0.5 cc. and a weaker but still noticeable reaction obtained with 0.2 cc. The possibility that substances like histamine were formed by the bacteria and accounted for the uterine reaction can be excluded by the failure of this material to affect the normal uterus. Whatever may have been the reason for this result, whether dialysis had anything to do with it or whether it means simply that the bacterial growth had destroyed the tuberculin substances while leaving the anaphylactinogens intact, it seems a definite and sharp separation of the two functions.

III.

Difference between Infected Animals and Those Treated with Dead Bacterial Substances.

Neither chemical fractionations nor the diffusion experiments furnish absolute proof that the anaphylactinogen can be completely separated from the tuberculin active substances. Nevertheless,

both series of experiments point in this direction, and, taken together with the mutual independence of the two types of reaction in guinea pigs, render such a conception an extremely likely one.

However this may eventually prove to be, one fundamental fact remains definite; namely, that the proteose fraction alone can elicit the particular form of hypersensitiveness which we speak of as the tuberculin reaction; and that the potency of the proteose residue in this respect is but slightly less then that of the whole extract, in spite of the fact that proteins have been removed as completely as is possible by boiling with acid.

Assuming, then, that the tuberculin type of reaction is a response to a proteose antigen, while the anaphylactic reaction is associated with the proteins, we are still confronted with the puzzling fact that the proteose reaction occurs only in infected animals and cannot ordinarily be induced in animals treated and rendered anaphylactic by injections of dead bacterial substances.

It might be assumed that the living tubercle bacillus in contact with the animal tissues produces a sensitizing substance which is not present in culture fluids or in dead tubercle bacilli. This idea is rendered improbable by the consideration that, although we almost uniformly fail in sensitizing guinea pigs to the cutaneous test by prolonged treatment with concentrated culture fluids or with bacillus extracts, nevertheless, skin reactions can be elicited in tuberculous animals by the proper application of such substances.

Another possibility, however, is that the substance which sensitizes to the tuberculin reactions is actually represented in the various tuberculin preparations and tubercle bacillus extracts, but that the intermittent method of injection, which must necessarily be used in the preparation of guinea pigs or other animals, does not simulate the manner in which these substances are being constantly diffused out into the animal tissues from growing foci. The supposition that the difference may lie in the manner of sensitization, both as to the time factor and in regard to the quantitative relations, is suggested by such experiments as those of Krause who has shown that cutaneous hypersensitiveness coincides with the establishment of a focus, diminishes with the healing of the focus, and varies directly with the intensity of the disease. It is suggested that in specific phenomena

of hypersensitiveness, of the tuberculin, typhoidin, mallein types, etc., we may be dealing with antigens that are subject to laws of sensitization and antibody formation, quite different from those governing the phenomena of protein anaphylaxis; in which the hypersensitiveness is elicited only by an intensive and concentrated contact with the antigenic substances; and in which, soon after the stimulus is removed, the hypersensitiveness diminishes. This, at least, seems a logical conclusion in regard to guinea pigs.

It should also be borne in mind, as has been suggested by Krause, that a general tuberculin reaction blunts skin hypersensitiveness considerably, and that a similar blunting of both the anaphylactic and the skin hypersensitiveness has been noted by others, as well as by ourselves, in the late stages of a fatal tuberculosis in guinea pigs, observations which indicate a reaction at least analogous to antigen-antibody reactions in general.

These considerations suggested to us the possibility that, although we might be giving the animals which we had treated with bacterial extracts some of the material which sensitizes them in the course of infection, we were not, perhaps, administering to them a sufficient amount of this proteose-like substance and were not giving it with the continuity with which it passes into the circulation of an animal suffering from an active process.

In order to obtain some light upon this, we carried out a number of experiments as follows:

Tubercle bacillus cultures were grown on 5 per cent glycerol peptone broth. When the growth had reached the size of a silver dollar or slightly more, but one-quarter of this growth was carefully lifted into another flask containing 100 cc. of similar glycerol broth, and the remaining three-quarters was killed at 80°C. for ½ hour. This dead growth, at least three-quarters of the original growth, was now washed several times with salt solution and infused in a flask containing 100 cc. of broth of the same lot as that on which the living one-quarter had been inoculated. The two flasks were then put into the incubator, and after the 2nd day skin reactions were done every day with the fluids from these flasks on tuberculous guinea pigs.

Fig. 8 shows the results. The reaction marked 1 on this figure is the reaction obtained with 0.1 cc., intracutaneously injected, of a 1:3 dilution of the 4 day growth in a highly sensitive guinea pig. The

reaction marked 2 shows the corresponding reaction carried out with the broth in which the dead tubercle bacilli, three times greater in bulk than the living ones, had been infused for the same length of time and used in the same dilution.

In order to eliminate any obstruction to diffusion which might have been brought about in the dead culture by the fact that heat was used in the killing, we did other experiments in which 0.5 per cent carbolic acid was added to the culture material to kill it.

Such an experiment is the following one. In this case a living growth about 1.2 cc. in diameter was floated on a flask of 100 cc. of glycerol broth and the remainder, about four times this amount, was submerged by shaking in 100 cc. of a similar broth flask, and 0.5 per cent carbolic acid added. On the 6th day reactions were done on tuberculous guinea pigs and on a control with the results shown in Table III.

TABLE III.

Skin Reactions Obtained with Material Killed with Carbolic Acid.

Broth.	Dilution.	Results with tuberculous guinea pigs.			Results with normal control
		No. 19.	No. 20.	No. 21.	
From living bacilli.	1:10	+	++	++	−
	1:20	+	+	±	−
From killed bacilli.	1:10	+	+	−	−
	1:20	±	±	−	−

Reactions of this type would indicate that the materials which caused skin reactions of the tuberculin type were being constantly diffused out from the growing cultures, while a limited amount only could be extracted from dead tubercle bacilli.

It is not impossible also that such a conception might indicate that these proteose-like materials were constantly being produced from the more complex material which we have spoken of tentatively as nucleoproteins, which represent the bulk of the soluble bacterial constituents and that this is the reason why we have never been able to free these acid-precipitable substances of their skin reaction capacity.

With this in mind we have recently treated four guinea pigs with large amounts of the so called nucleoproteins, giving each animal

four intraperitoneal injections, each of which represented at least 50 cc. of original extract. And in these animals, 14 days after the last injection, we saw the first hopeful indications of positive skin reactions of the tuberculin type, artificially induced. These experiments will be continued.

IV.

DISCUSSION AND CONCLUSIONS.

The work reported in the preceding sections justifies, we think, a number of definite conclusions. In addition to this, some of the experiments indicate a line of thought which may lead to considerable alteration in our conceptions, both of phenomena of bacterial hypersensitiveness and of infection.

1. In guinea pigs two fundamentally different types of intradermal reactions may be observed. One of these is the immediate, transitory reaction which develops in animals sensitized against proteins (horse serum, etc.) and may be regarded as one of the manifestations of general protein hypersensitiveness, or anaphylaxis; the other is the tuberculin type of skin reaction which develops more slowly, leads to a more profound injury of the tissues and is independent of anaphylaxis as ordinarily conceived.

2. The tuberculin type of hypersensitiveness (as well as probably the typhoidin, mallein, abortin reactions, etc.) does not develop at all in guinea pigs sensitized with proteins, like horse serum, etc. While this form of hypersensitiveness may eventually be induced with materials not bacterial in origin, it has been observed up to date only as a reaction of bacterial infection.

3. Methods of treatment with protein material from bacterial cultures which sensitize guinea pigs to anaphylactic reactions with the bacterial extracts, do not sensitize them to the tuberculin type of reaction. Such sensitization is easily accomplished only by infecting the animals with living organisms. No reliable method of sensitizing guinea pigs to such reactions with dead bacterial material has as yet been worked out, though a few hopeful experiments have been obtained with massive injections of large amounts of the acid-precipitable substances (nucleoproteins?) from bacterial extracts.

4. In animals made hypersensitive to the tuberculin type of reaction by infection with living bacteria, the reaction may be elicited by intradermal injections of bacterial extracts from which all coagulable proteins, nucleoproteins, and Bence-Jones proteins have been removed, as well as this can be done by boiling with acid, etc. This proteose residue alone suffices to elicit such reactions. The exact chemical nature of the so called proteose residue must be further studied and analyzed when we have had opportunity to produce bacterial extracts in large quantity.

These points seem incontrovertible on the basis of our own experiments, as well as those of other workers.

There thus seem to develop two definite forms of hypersensitiveness in guinea pigs infected with bacteria, typical anaphylaxis in which the protein material of the bacterial cells is concerned, which develops late and which can be induced by repeated injections of dead bacterial material, and a hypersensitiveness to non-protein constituents which differs from the former, both in the laws that govern sensitization and in the manifestations which follow injections into the sensitized animals.

While there is virtual agreement among immunologists concerning the essential mechanism of protein anaphylaxis, its dependence upon an antigen-antibody reaction, and the dominating rôle played by the sessile antibodies, the mechanism of hypersensitiveness to tuberculin and similar bacterial substances is still a problem of much uncertainty.

The most striking difference between the two phenomena lies, as we have seen, in the criteria of sensitization, in that hypersensitiveness to the tuberculin type of reaction can hardly ever be induced by any of the ordinary methods of preparation with the constituents of dead bacteria, but develops promptly (7 to 10 days) in the course of actual infection with living organisms.

The considerable specificity of such reactions forces the conclusion that the sensitizing substance must, in some way, be derived from the infecting microorganisms.

The idea that the failure of sensitization with dead culture materials is perhaps due to the elaboration in the body of infected animals of bacterial products not represented in extracts of test-tube cultures is rendered unlikely by the fact that in the tuberculin-sensitive,

infected animals, we can produce the reactions by the application of such dead extracts. It is neither logical nor in keeping with biological experience to assume that one substance will sensitize to reaction with another. This mistake was made early in the study of anaphylaxis in another connection and caused considerable delay of progress.

Krause has shown that tuberculin sensitiveness may be blunted in infected animals by massive, but sublethal injections of tuberculin, and we have obtained some indications of the same thing. Moreover, others as well as ourselves have seen tuberculin reactivity decline in guinea pigs and in man in the stages of very severe infection. These facts would eliminate any assumption of mere cumulative injury as explaining this type of reaction, and stamp it as a mechanism at least analogous to ordinary anaphylaxis.[7]

The only remaining possibility to explain the difference between infected animals and those treated with dead bacterial constituents would be to assume that the difference must lie in the manner in which the sensitizing substance is administered to the animals, and that sensitization with the proteose residue materials depends upon criteria of sensitization differing in regard to the time and quantity factors from those governing protein sensitization. If one considers the relatively simpler chemical structure and perhaps physically greater diffusibility of the materials concerned in this reaction, one might readily expect such differences in the methods needed for sensitization.

In keeping with such a line of reasoning our experiments have shown that the tuberculin active materials are constantly and rapidly being diffused out into the culture fluid from growing organisms, in quantities greater than can be extracted from similar amounts of the dead bacteria. It seems reasonable to assume from this that the same thing may happen in the animal body harboring a growing focus. And it would seem quite likely that the association of the tuberculin type of reaction with actual infection may depend upon the fact that sensitization to these non-protein substances depends upon a constant steady absorption of large amounts of the material.

Moreover, the only hopeful experiments on the artificial production of tuberculin sensitiveness in guinea pigs obtained by us were those

[7] Direct attempts to show such cumulative toxic action have failed in our hands.

in which massive doses of the nucleoprotein material injected into guinea pigs gave rise to a moderate skin sensitiveness.

Does the so called proteose residue form antibodies, and, if so, are substances analogous to antibodies involved in the tuberculin type of hypersensitiveness?

The failure to transfer passively this form of hypersensitiveness to normal animals with the blood and tissues of tuberculin-sensitive ones would suggest that no antibodies are involved. But this is not conclusive on the basis of available experimental facts. We are inclined to believe that antibodies of a sort are involved, for the following reasons: (*a*) In our experiments with the uteri of highly sensitive extract-treated guinea pigs and of tuberculous guinea pigs, we have occasionally had positive reactions when the proteose residue alone was used. (*b*) We believe that these proteose substances are entirely analogous to the substances studied by Avery and Dochez (22) in the urine and blood of typhoid and pneumonia patients. They obtained precipitin reactions against homologous immune sera with the urine of infected cases concentrated by evaporation after boiling with acetic acid to remove coagulable proteins. (*c*) Petroff, with whom we discussed this proteose residue early in our work, has produced it, and tells us that he has obtained precipitin reactions with it by titrating it against the serum of a sheep treated for a long time with tubercle bacillus products.

In suggesting an antibody response to a non-protein antigen we are aware that we are opposing what has been regarded as a well established doctrine in immunity; this is justified, or at least mitigated, we believe, by the consideration that reactions of the antigen-antibody type are the only explanation of specificity; and tuberculin, mallein, and typhoidin reactions are to a considerable degree specific. If such reaction bodies cannot be produced by precisely the same methods of administration as to time and quantity which are successful in calling forth protein antibodies, this should not astonish us, since, after all, the substances that we are dealing with are simpler in chemical structure than are the proteins, and physically are probably of relatively greater diffusibility. It may be that the greater diffusibility of the proteose-like substances transfers much of the actual reaction phenomena to an intracellular location, and that this

to some extent influences the presence of circulating antibodies. It may also be that these more diffusible non-protein antigens are more rapidly eliminated from the animal body than are the proteins. Indeed, the above mentioned observations of Avery and Dochez, and the recent work of Wildbolz (23), Lanz (24), Imhof (25), and Gibson and Carroll (26), who demonstrated tuberculin active antigens in the urine of active cases, would corroborate such a view. The evidence available at the present time, however, concerning antibody formation to these non-protein substances is, we recognize, largely indirect, at least as far as our own work is concerned, and we present it in the present connection purely as a working hypothesis.

Finally, perhaps the most important theoretical consideration indicated by our experiments is the following. We have in the tuberculin reaction a form of hypersensitiveness which seems to be (in guinea pigs, at least) analogous entirely to the typhoidin reaction, the mallein reaction, and the abortin reaction. Whenever reactions of this type have been carefully studied, whatever the bacteria involved, they have been associated with infection as in tuberculosis, and have been followed by analogous clinical manifestations. It would seem perhaps that we are dealing with a law applicable to bacterial infection in general.

It would appear that certain non-coagulable substances of uncertain chemical constitution are being constantly elaborated in the course of bacterial growth, and passed into the circulation of infected animals. As a result of this, infected animals become sensitized to these heat- and acid-resistant materials, in tuberculosis in the course of 1 to 2 weeks, in the case of more rapidly growing bacteria perhaps sooner. Early in the course of infection, the animal becomes sensitized and subsequently the further elaboration and distribution of these materials from the bacterial focus plays a fundamental part in the injury of the animal. These proteose-like substances, like tuberculin, possessing but slight toxicity for the normal animal, become highly toxic to the sensitized one. Thus, these substances, while not being true exotoxins in the ordinary sense, would still represent a highly toxic bacterial product comparable in its injurious effect to toxins when produced in the body of an animal thus sensitized.

If there is any value in these deductions the attention of bacteriologists should be turned to the non-protein constituents of bac-

terial cells in their further immunological studies, as well as to the protein materials.

It is obvious that the next step in our investigations must consist in producing the non-coagulable material from bacterial extracts in considerable quantity, to determine their antibody-forming properties in detail, and elucidate, if possible, the laws which govern sensitization with them. This work has been begun, but it has seemed advisable to publish this as far as we have gone because it will take a long time before it can be completed.

BIBLIOGRAPHY.

1. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1917, xxvi, 411.
2. Fleischner, E. C., Meyer, K. F., and Shaw, E. B., *Am. J. Dis. Child.*, 1919, xviii, 577.
3. Moss, W. L., *J. Am. Med. Assn.*, 1910, lv, 776.
4. Knox, J. H. M., Moss, W. L., and Brown, G. L., *J. Exp. Med.*, 1910, xii, 562.
5. Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, xxvii, 341.
6. Mackenzie, G. M., and Leake, W. H., *J. Exp. Med.*, 1921, xxxiii, 601.
7. Calmette, A., L'infection bacillaire et la tuberculose, Paris, 1920.
8. Römer, P. H., *Berl. klin. Woch.*, 1909, xlvi, 813; *Deutsch. med. Woch.*, 1909, xxxv, 245.
9. Baldwin, E. R., *J. Med. Research*, 1910, xxii, 189.
10. Austrian, C. R., *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 1.
11. Krause, A. K., *Am. Rev. Tuberc.*, 1917–18, i, 65.
12. Bail, O., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 470.
13. Helmholz, H. F., *Z. Immunitätsforsch., Orig.*, 1909, iii, 371.
14. Gay, F. P., and Claypole, E. J., *Arch. Int. Med.*, 1914, xiv, 671.
15. Meyer, K. F., and Christiansen, C. R., *Collected Reprints from the George Williams Hooper Foundation for Medical Research*, 1916–17, ii, 1.
16. Meyer, K. F., *Collected Reprints from the George Williams Hooper Foundation for Medical Research*, 1916–17, ii, 68.
17. Nichols, H. J., *J. Exp. Med.*, 1915, xxii, 780.
18. Weil, R., *J. Am. Med. Assn.*, 1917, lxviii, 972.
19. Löwenstein, E., and Pick, E. P., *Biochem. Z.*, 1911, xxxi, 142.
20. Brown, W., *Biochem. J.*, 1915, ix, 591.
21. Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.
22. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
23. Wildbolz, H., *Cor.-Bl. schweiz. Aerzte*, 1919, xlix, 793.
24. Lanz, W., *Schweiz. med. Woch.*, 1920, l, 321.
25. Imhof, O., *Schweiz. med. Woch.*, 1920, l, 1033.
26. Gibson, C. B., and Carroll, W. E., *J. Am. Med. Assn.*, 1921, lxxvi, 1381.

EXPLANATION OF PLATES.

PLATE 40.

FIG. 1. Guinea Pig 22. Another instance of positive skin reaction after 9 days, with negative uterine test. This animal was of the same lot as Guinea Pig 23.

FIG. 2. This record is a simultaneous record of two guinea pigs inoculated on November 21, 1920, with large amounts (2 cc.) of a suspension of a young tubercle bacillus culture (human). No. 23 (upper curve) inoculated intraperitoneally, and No. 24 (lower curve) subcutaneously. On December 1, 1920, skin reactions were done with two types of extract, one a sodium carbonate extract and the other a sodium hydroxide extract. No. 23, which was apparently weaker and emaciated, showed practically no reaction in either place, whereas No. 24, to our surprise, showed a definite reaction at both points. In spite of the differences in skin reactions, it will be seen that the uterine reactions in both guinea pigs were negative. The two uteri were put into the same bath simultaneously in order to subject them to exactly the same conditions. The negative reaction of the uterus was confirmed with the other horns of both guinea pigs. The surprising thing is the earliness with which the skin reaction appeared, which tends to confirm one of a series of observations that the skin reaction appears much earlier than the uterine reaction.

FIG. 3. Guinea Pig 25. Inoculated subcutaneously with tubercle bacilli on November 22, 1920. Showed that powerful anaphylactic reaction may develop within 3 weeks after a heavy inoculation with tubercle bacilli. This, however, is unusually early. Skin reaction in this guinea pig was positive.

PLATE 41.

FIG. 4, *a* and *b*. (*a*) Guinea Pig 26. (*b*) Guinea Pig 27. Two guinea pigs of a series injected every other day intraperitoneally with unfiltered alkaline extract of powdered tubercle bacilli. Last injection given November 29, 1920. No. 26 gave a negative skin reaction on December 6, but a positive uterine reaction on December 7. A similar uterine test with another guinea pig (No. 28) showed a very moderate uterine reaction, and this, with other experiments, indicates that the uterine or anaphylactic reaction does not develop until at least 8 or 9 days after the last preparatory injection. Guinea Pig 27 in the second record was treated exactly like the preceding animal, but here the tests were not done until December 21, slightly over 3 weeks since the last extract injection. Here again the skin reaction was entirely negative and the uterine test very much more powerful. The uterine reaction does not develop to its fullest extent until about 2 or 3 weeks after the last injection.

FIG. 5, *a* to *c*. (*a*) Normal guinea pig. (*b*) Guinea Pig 29. (*c*) Guinea Pig 30. The records show a failure of reaction of the uteri of two different sensitized guinea pigs (Nos. 29 and 30) after the addition of the proteose residues alone, but

powerful reactions when, following this, whole extracts were introduced into the bath. The record at the top is a normal control with the same extracts, showing that the reactions below are specific.

PLATE 42.

FIG. 6, *a* and *b*. This is one of two preparations in which the materials used consisted of two fractions obtained from the same 50 cc. of a 0.02 per cent alkaline extract of powdered tubercle bacilli. The extract was centrifuged until clear, the supernatant fluid acidified to pH 4.5, which was the point at which the flocculent precipitate did not increase. This was centrifuged away and spoken of as nucleoprotein. This nucleoprotein was twice redissolved in more than 50 cc. of alkaline salt solution and reprecipitated with acid, the material being centrifuged in each case in order to purify as much as possible. The supernatant fluid was then boiled in the acid condition to remove coagulable proteins and twice filtered through a Berkefeld filter. It will be noted that in looking at these two preparations together after testing against different strips of the same sensitized guinea pig uterus (No. 31), the total amounts of the two fractions gave the same results, though in different proportions, an additional proof that cumulative effects had nothing to do with the results.

FIG. 7. February 18. The sensitized guinea pig treated with Material B_2 which, as stated before, gave definite though somewhat weakened skin reactions. Here, 6 cc. on the outside of the bag, which still gave skin reactions, gave no anaphylactic reaction. The high rise is probably due to the fact that this uterus was very irritable. That the rise was not an anaphylactic reaction is apparent from the immediate return to the base-line, and the fact that there was no apparent desensitization to the subsequent addition of 2 cc. from the inside of the bag. Dialysate was positive on skin and negative on uterus.

PLATE 43.

FIG. 8. Drawing of skin reactions on a tuberculous guinea pig, carried out with broth from a culture of living tubercle bacilli 4 days old, 1:3 dilution, at the point marked 1, and a similar reaction with a similar amount of broth from a killed culture, 1:3 dilution, at the point marked 2.

FIG. 1.

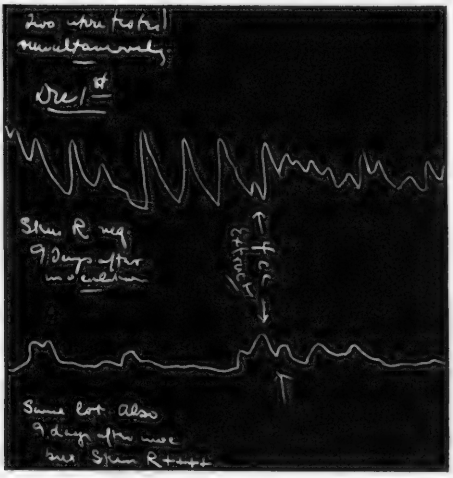


FIG. 2.

FIG. 3.

(Zinsser: Tuberculin reaction.)

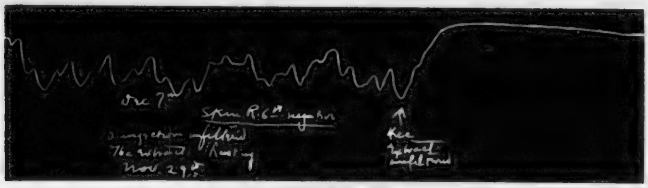


FIG. 4, *a*.

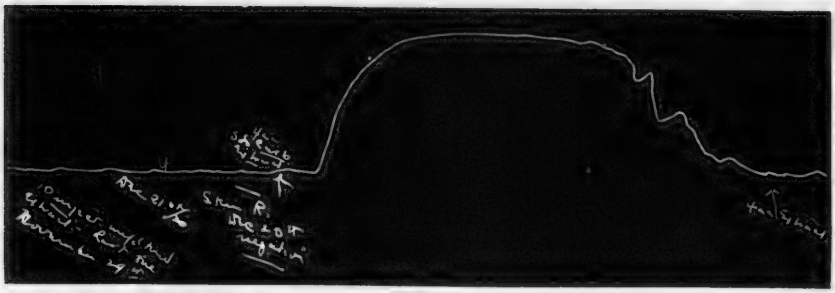


FIG. 4, *b*.

FIG. 5, *a*.

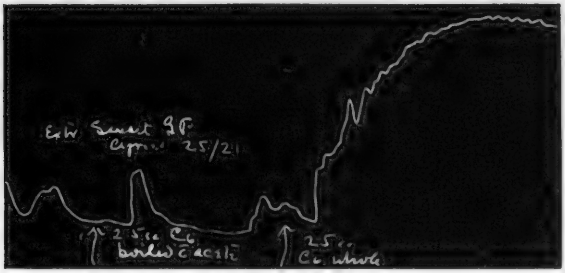


FIG. 5, *b*.

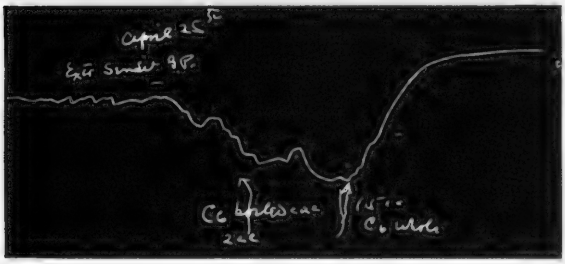


FIG. 5, *c*.

(Zinsser: Tuberculin reaction.)

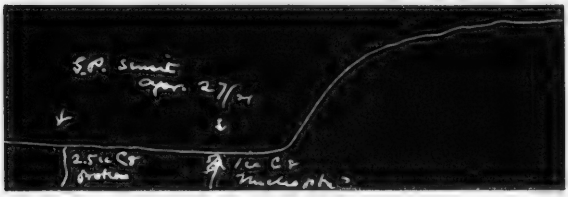


FIG. 6, *a*.

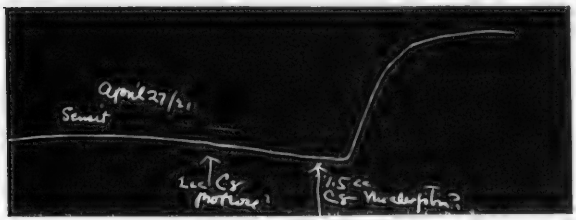


FIG. 6, *b*.

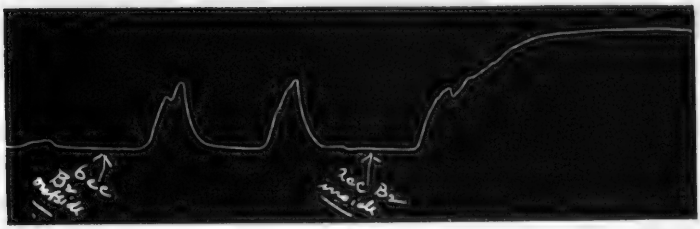


FIG. 7.

(Zinsser: Tuberculin reaction.)

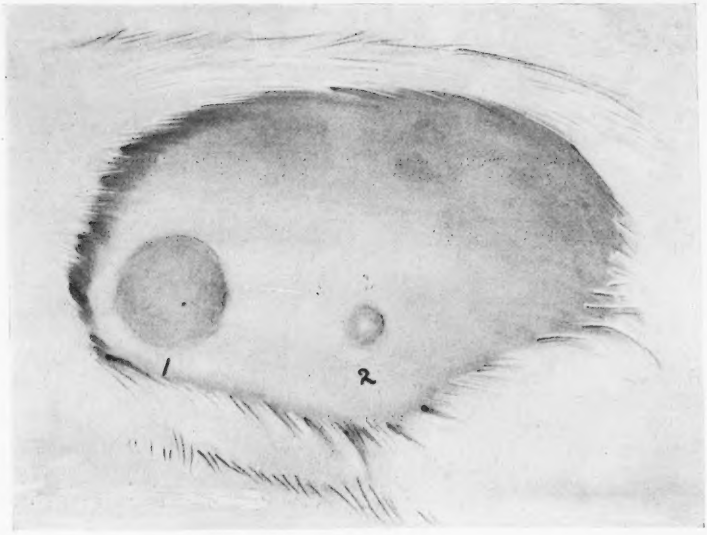


FIG. 8.

(Zinsser: Tuberculin reaction.)